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THE UNIVERSITY OF ALBERTA

THE HYPOTHALAMIC REGULATION OF GROWTH HORMONE SECRETION IN THE GOLDFISH, *Carassius auratus* L.

by

TRACY A. MARCHANT

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA

FALL, 1987

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled THE HYPOTHALAMIC REGULATION OF GROWTH HORMONE SECRETION IN THE GOLDFISH, Carassius auratus L. submitted by TRACY A. MARCHANT in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

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ABSTRACT

In the present study, the hypothalamic peptide somatostatin-14 (SRIF-14) was found to rapidly inhibit GH secretion *in vitro* in a potent and dose-dependent manner, demonstrating that SRIF-14 acts directly at the level of the pituitary to alter GH release in the goldfish. Additional *in vitro* experiments suggest that SRIF receptors on the goldfish pituitary are specific for molecules containing the SRIF-14 sequence. Evidence of a relationship between endogenous SRIF and GH secretion was provided by experiments demonstrating that the amount of immunoreactive SRIF (irSRIF) in the pituitary, hypothalamus and telencephalon varies in an inverse relationship to serum GH levels throughout the year. Brain lesion experiments provided direct evidence that the preoptic area is the origin of an inhibitory somatostatinergic projection innervating the pituitary. These results support the hypothesis that SRIF-14 or a very similar molecule functions as a GH release-inhibitory factor in the goldfish.

Gonadotropin (GTH)-releasing hormone (GnRH) was found to elevate serum GH levels in both female and male goldfish, and repeated injections of GnRH were found to accelerate body growth. *In vitro* experiments provide strong evidence that GnRH acts directly at the level of the pituitary to stimulate GH secretion, and suggest that the receptor mechanisms mediating the stimulatory actions of GnRH may be similar for GH and GTH secretion. However, *in vivo* and *in vitro* experiments provide evidence of separate release-inhibitory factors for GH and GTH secretion. For example, SRIF-14 was found to inhibit

GnRH-induced GH secretion in vitro but did not influence GTH secretion. Conversely, apomorphine (an agonist of the teleost GTH release-inhibitory factor dopamine) suppressed GnRH-induced GTH secretion but did not influence the GnRH stimulation of GH secretion.

Based on the results outlined above, it is hypothesized that the secretion of both GH and GTH in the goldfish are regulated by the hypothalamus, at least in part, through a common releasing factor - GnRH. However, evidence suggests that the hypothalamic factors inhibiting GH and GTH secretion in the goldfish are separate and distinct.

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1. GENERAL INTRODUCTION

Based on anatomical and experimental evidence, it was hypothesized in the late 1940's that the secretion of mammalian adenohypophyseal hormones was regulated by the central nervous system (CNS) through the release of neurohumoral substances into the hypothalamo-hypophyseal blood portal vessels [Green and Harris, 1947; Harris, 1948]. According to this hypothesis, substances would be released from neurosecretory neurons in the hypothalamus and travel via the portal blood to influence the secretion of hormones from the anterior pituitary gland. Extensive evidence has accumulated to provide support for this concept in vertebrates, culminating in the isolation and characterization of several substances qualifying as hypothalamic hypophysiotropic factors [for review: Donovan, 1978; Halász, 1985; McCann, 1980; Peter, 1986; Vale _et_ _al_., 1977].

Two peptides influencing the secretion of growth hormone (GH) from the pituitary have been characterized from the mammalian hypothalamus. The first peptide is a 14 amino acid molecule originally isolated from ovine hypothalami, and characterized by its ability to inhibit GH secretion from the rat pituitary [Brazeau _et_ _al_., 1973]. This GH release-inhibitory peptide is called somatostatin (SRIF). The identical peptide was subsequently isolated from porcine hypothalami [Schally _et_ _al_., 1976] and appears to be identical in all of the mammalian species studied to date [Patel and Srikant, 1986]. The second peptide, GH-releasing hormone (GHRH), was isolated more recently, although the search for this substance actually spanned three decades of research

[Frohman and Jansson, 1986]. GHRH was originally isolated from human pancreatic islet tumors removed from acromegalic patients [Guillemin *et al*., 1982; Rivier *et al*., 1982], although the identical molecule was subsequently isolated from the human hypothalamus [Ling *et al*., 1984]. Molecules similar in structure to the human GHRH have also been isolated from murine [Spiess *et al*., 1983], porcine [Bohlen *et al*., 1983], bovine [Esch *et al*., 1983], ovine [Brazeau *et al*., 1984] and caprine [Brazeau *et al*., 1984] hypothalami.

A large number of studies have confirmed the role of SRIF and GHRH in the neuroendocrine regulation of GH secretion in mammals [for review: Arimura and Culler, 1985; Frohman and Jansson, 1986; Patel and Srikant, 1986]. Both peptides have been identified in the mammalian hypothalamus and hypothalamo-hypophyseal portal blood, and have been found to alter GH secretion *in vivo* and *in vitro*. Furthermore, passive immunoneutralization of the endogenous peptides through the administration of antisera or monoclonal antibodies specific for SRIF or GHRH result in the elevation or reduction, respectively, of circulating GH levels. Finally, receptors specific for each of these peptides have been demonstrated in membrane preparations of the mammalian pituitary, and studies suggest that GHRH stimulates GH secretion through activation of the adenylate cyclase-cAMP system, whereas SRIF appears to inhibit GH secretion, at least in part, through opposing actions on the cAMP system as well as on processes subsequent to cAMP production [Frohman and Jansson, 1986; Patel and Srikant, 1986].

The secretion of GH in mammals occurs in an episodic pulsatile manner, and, consequently, the circulating profile of GH is characterized by rapid increases in circulating GH levels followed by return to near undectable levels in the intervening periods [Martin, 1979; Arimura and Culler, 1985]. Based on studies primarily in the rat, Martin and his colleagues originally proposed that the episodic secretion of GH _in vivo_ resulted from the phasic release of both SRIF and GHRH from the hypothalamus [Martin, 1979; Tannenbaum, 1985]. According to this hypothesis, SRIF would be released tonically from the hypothalamus with an additional surge of SRIF release resulting in low circulating levels of GH during the intervening periods. GHRH would also be released phasically, but 180° out of phase with SRIF, to stimulate a pulse of GH release during the attenuation of the inhibitory SRIF influence. Several lines of evidence provide support for this hypothesis [Arimura and Culler, 1985; Martin, 1979; Patel and Srikant, 1986; Tannenbaum, 1985], and a recent study measuring the levels of SRIF and GHRH in hypophyseal portal blood of rats [Plotsky and Vale, 1985] has demonstrated surges of GHRH in the portal blood occurring concurrently with a decrease in the concentration of SRIF. Thus, the phasic, integrated release of SRIF and GHRH from the hypothalamus appears to be required to generate the normal profile of circulating GH levels in mammals.

Several neurotransmitter systems and brain peptides also influence GH secretion in mammals through actions on the CNS [Arimura and Culler, 1985; Martin, 1979, 1980]. An α-adrenergic system stimulating the normal pattern of GH release appears to be of primary importance in mammals, although various other neurotransmitters including dopamine, a β-adrenergic system, serotonin, acetylcholine, GABA, histamine and various other peptides have also been shown to alter GH secretion [Arimura and Culler, 1985; Hall _et al_., 1986]. The majority of evidence indicates that these substances act through the hypothalamus to alter the release of SRIF and/or GHRH from the hypothalamus [Arimura and Culler, 1985; Martin, 1980]. For example, studies indicate that the α-adrenergic system may stimulate GH release through a dual action on the hypothalamus: the stimulation of GHRH release [Katakami et al., 1984; Miki et al., 1984] as well as a reduction in SRIF secretion [Chihara et al., 1984; Ishikawa et al., 1983]. Although it is generally assumed that neurotransmitters act via the hypothalamus or other CNS areas to alter GH secretion in mammals, recent evidence indicating the presence of a β-adrenergic system inhibiting [Perkins et al., 1983] and a dopaminergic system stimulating [Serri et al., 1987] GH release from perifused rat anterior pituitary cells provides the possibility that some neurotransmitters may also act directly at the level of the pituitary.

Shortly after its isolation from the mammalian hypothalamus [Brazeau et al., 1973], SRIF was also found to be present in the D cells of the pancreatic islets [Arimura et al., 1975; Dubois, 1975]. Subsequent studies have demonstrated that SRIF is widely distributed in a variety of mammalian tissues, including extra-hypothalamic areas of the CNS, the peripheral nervous system, and the gastrointestinal tract [for review: Arimura, 1981; Arimura and Fishback, 1981; Reichlin, 1983a, 1983b]. In keeping with this wide tissue distribution, SRIF also has a diverse spectrum of actions in mammals. In addition to inhibiting GH secretion from the pituitary, SRIF also inhibits thyrotropin (TSH) secretion, and it is now generally accepted as an important regulator of TSH secretion under physiological conditions [Patel and Srikant, 1986]. Under certain conditions, SRIF has also been reported to inhibit the secretion of prolactin (PRL) and other pituitary hormones [Patel and Srikant, 1986]. Within the CNS, evidence indicates that SRIF may function as a neuromodulator or neurotransmitter [Arimura, 1981; Reichlin, 1983b]. SRIF secreted by the D cells is thought to act in a paracrine or endocrine fashion within the pancreas to inhibit the secretion of pancreatic hormones. SRIF has also been shown to suppress various endocrine and exocrine functions in other areas of the mammalian gastrointestinal tract [Arimura, 1981; Arimura and Fishback, 1981; Reichlin, 1983b]. Finally, SRIF, originating primarily from peripheral tissues, is known to be present in the mammalian circulation [Patel et al., 1981], and it has been suggested that peripherally-released SRIF may also influence pituitary hormone release [Patel and Srikant, 1986]. Thus, in addition to its actions as a hypophysiotropic factor, SRIF displays a wide diversity of biological actions in mammals, many of which are related to the regulation of nutrient homeostasis and metabolic status; consequently, it is possible that SRIF may influence growth in mammals through actions at several levels, only one of which is the secretion of GH [Borer et al., 1983].

In contrast to mammalian species, fewer studies have investigated the role of the hypothalamus in the regulation of GH secretion in other vertebrates species, and the majority of these studies have been focused on avian species [Hall et al., 1986]. In birds, a well-developed hypothalamo-hypophyseal portal system is present [Schriebman, 1986], and evidence indicates that at least three hypothalamic peptides are involved in the regulation of GH secretion [Hall et al., 1986; Harvey, 1983; Scanes et al., 1986]. SRIF-14 identical in structure to the mammalian molecule has been isolated from birds [Spiess et al., 1979;

Hasegawa *et al.*, 1984], and has been shown to inhibit GH secretion *in vivo* and *in vitro* in a variety of avian species [Hall *et al.*, 1986; Harvey, 1983; Scanes *et al.*, 1986]. Mammalian GHRH stimulates GH secretion in birds, and GH secretion is proposed to be regulated, at least in part, through the release of a molecule similar in structure of mammalian GHRH [Hall *et al.*, 1986; Scanes *et al.*, 1986]. Strong evidence indicates that TSH-releasing hormone (TRH) is also a potent stimulator of GH secretion in birds [Hall *et al.*, 1986; Harvey, 1983; Scanes *et al.*, 1986]. Several neurotransmitters and brain peptides also influence GH secretion in birds, primarily through central actions mediated by hypothalamic peptides [Hall *et al.*, 1986]. However, an adrenergic system inhibiting GH release from chicken pituitary cells *in vitro* has been reported, and it has been suggested that epinephrine may also qualify as a hypohysiotropic factor in this species [Scanes *et al.*, 1986].

The hypothalamic regulation of GH secretion in poikilothermic vertebrates has not been extensively studied and, consequently, is poorly understood compared to mammalian and avian species [Ball, 1981; Hall *et al.*, 1986; Peter and Fryer, 1983]. The anatomical relationship between the brain and pituitary in lower vertebrates does provide the possibility for hypothalamic regulation of adenohypophyseal function. In the agnathans (lampreys and hagfish), the brain is separated from elements of the anterior pituitary gland by a sheet of poorly vascularized connective tissue, with no identifiable vascular or neural connections between the two components [Ball, 1981; Gorbman, 1980; Schriebman, 1986]. This is considered to represent the most primitive vertebrate brain-pituitary relationship [Gorbman, 1980], and studies indicate that the agnathan brain may influence pituitary function through the diffusion of neuropeptides or other substances across the connective tissue layer [Tsukahara et al., 1986; Schriebman, 1986]. A well-developed hypothalamo-hypophyseal portal system, homologous to that of birds and mammals, first appears in the cartilaginous fishes, and is also present in the more primitive bony fishes (holosteans and ascipenseroids), lungfishes, the coelacanth, amphibians and reptiles [Ball, 1981; Schriebman, 1986]. However, in the most advanced actinopterygian fishes, a unique brain-pituitary relationship has evolved. In general, a true median eminence or portal system is lacking in teleost fishes; instead, the neurohypophysis interdigitates with the anterior pituitary, and hypophysiotropic nerve fibers terminate on the basal lamina or actually penetrate into the pars distalis to provide a direct innervation of the endocrine cells [Ball, 1981; Peter and Fryer, 1983; Schriebman, 1986]. One of the consequences of this form of brain-pituitary association in teleosts, is that the anterior pituitary is directly innervated by both petidergic and aminergic fibers from the hypothalamus [Kaul and Vollrath, 1974; Peter and Fryer, 1983], resulting in the possibility that substances such as catecholamines may also function as hypophysiotropic factors in teleosts [Peter et al., 1986].

In spite of variations in the anatomical relationship of the brain and pituitary in various vertebrate classes, it is generally accepted [Ball, 1981; Peter and Fryer, 1983; Schriebman, 1986] that the brain regulates pituitary hormone secretion in lower vertebrates in a manner essentially similar to that orginally proposed for mammalian species [Green and Harris, 1947]. Recently, SRIF-14 identical in structure to the molecule found in mammalian and avian species has been isolated from tissues of an elasmobranch [Conlon et al., 1985] and three teleost species [Andrews and Dixon, 1981; Noe et al., 1979; Plisetskaya et al., 1986], suggesting that the structure of at least one hypophysiotropic peptide has been fully conserved throughout vertebrate evolution. Other studies using various immunological techniques have demonstrated the presence of substances immunologically related to several mammalian hypothalamic peptides in brains from representatives of all classes of lower vertebrates [Crim and Vigna, 1983; Peter, 1986]. The presence in lower vertebrates of molecules similar or identical in structure to mammalian hypothalamic peptides has led to the speculation that the hypophysiotropic actions of these molecules may have developed early in vertebrate evolution [Jackson, 1986].

Although it is generally accepted that the hypothalamus regulates GH secretion in poikilothermic vertebrates [Ball, 1981; Hall et al., 1986; Peter and Fryer, 1983], studies investigating the role of the hypothalamus in the regulation of GH secretion are actually few in number in most of the classes of lower vertebrates, and are completely lacking in representatives of the agnathans, cartilaginous fish and primitive bony fishes. Much of the information currently available regarding the hypothalamic control of GH release in lower vertebrates has come from studies examining the influence of crude extracts of the hypothalamus on GH release *in vitro* [Ball, 1981; Hall and Chadwick, 1978, 1979] in which the identity of the active substance(s) in the extracts could not be determined. Studies examining the influence of synthetic mammalian hypophysiotropic peptides on GH release in lower vertebrates [Cook and Peter, 1984; Fryer et al., 1979; Hall and Chadwick, 1983, 1984; Peter et al., 1984; Rivas et al., 1986; Wigham and Batten, 1984], and studies identifying specific areas of the hypothalamus involved in regulating GH secretion [Cook and Peter, 1983; Fryer, 1981; Peter and McKeown, unpublished results cited in Peter and Fryer, 1983; Pickford *et al*., 1981] are limited to a few species. Immunocytochemical studies examining the distribution of peptides in the CNS of lower vertebrates are also limited, and the functional significance of many of the findings from these studies remains unclear [Peter, 1986]. Therefore, additional studies in a number of species are required before the involvement of hypothalamic hypophysiotropic factors in the regulation of GH secretion can be ascertained in lower vertebrates. Until the results of such studies are available, the evolutionary pattern of the hypothalamic control of GH secretion in vertebrates will also remain obscure.

In this thesis, results of experiments examining the role of the hypothalamus in regulating GH secretion in a teleost species, the goldfish (*Carassius auratus* L.) are presented. Several experimental approaches were used to examine this aspect of GH physiology in the goldfish. In Chapter 2, the influence of various teleost and mammalian SRIF peptides on GH secretion *in vitro* was examined using fragments of the goldfish pituitary maintained in a perifusion system. The relationship between endogenous brain SRIF and the seasonal profile of circulating GH levels in the goldfish was determined by measuring the amount of immunoreactive (ir) SRIF in the pituitary and various brain regions at several times throughout the year (Chapter 3). The hypothalamic origin of irSRIF fibers in the goldfish pituitary was also examined in Chapter 3 using brain lesioning techniques. In Chapter 4, results from *in vivo* experiments indicating that gonadotropin-releasing hormone (GnRH) elevates circulating GH levels in the goldfish are presented. The effect of GnRH on GH secretion *in vitro* was examined in Chapter 5, and compared to the influence of GnRH on gonadotropin secretion from perifused fragments of the goldfish pituitary. Chapter 6 examines the influence of an analog of GnRH on somatic growth in the goldfish. The influences of human GHRH and a carp GHRH-like peptide on GH secretion *in vivo* and *in vitro* in the goldfish are also studied (Chapter 7). Finally, the results obtained from the various experiments in this thesis are discussed (Chapter 8) in the context of developing a model outlining the major components involved in the hypothalamic regulation of GH secretion in the goldfish.

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2. THE INFLUENCE OF MAMMALIAN AND TELEOST SOMATOSTATINS ON THE SECRETION OF GROWTH HORMONE FROM GOLDFISH PITUITARY FRAGMENTS IN VITRO

2.1 INTRODUCTION

The growth hormone (GH) release-inhibitory factor somatostatin (SRIF) was originally isolated from the hypothalamus and pancreas of mammalian species [Arimura *et al*., 1975; Brazeau *et al*., 1973]. In mammals, a single precursor molecule, prosomatostatin (proSRIF), is present [Goodman *et al*., 1982; Shen *et al*., 1982]. A 14 amino acid peptide (SRIF-14) and a 28 amino acid peptide (SRIF-28) containing the SRIF-14 sequence at its carboxyl terminus are produced from the single mammalian proSRIF [Goodman *et al*., 1982; Millar *et al*., 1983; Shen *et al*., 1982]. A molecule identical in structure to SRIF-14 has since been isolated from tissues of several other vertebrate species, including two avian species [Hasegawa *et al*., 1984; Spiess *et al*., 1979], an elasmobranch (*Torpedo marmorata*) [Conlon *et al*., 1985], and three teleost species: the catfish (*Ictalurus punctata*) [Andrews and Dixon, 1981], the anglerfish (*Lophius americanus*) [Noe *et al*., 1979], and the coho salmon (*Oncorhynchus kisutch*) [Plisetskaya *et al*., 1986].

In contrast to the mammalian species, however, cDNA sequencing studies have identified two active genes encoding two distinct somatostatin precursors (proSRIF-I and proSRIF-II) in the pancreas of both catfish and anglerfish [Andrews and Dixon, 1981; Hobart *et al*.,

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1980; Magazin et al., 1982; Minth et al., 1982]. In both of these teleost species, SRIF-14 identical to the mammalian peptide is produced from proSRIF-I, whereas the peptide produced from proSRIF-II is structurally distinct from SRIF-14 [Andrews et al., 1984a, 1984b; Morel et al., 1984a; Hobart et al., 1980; Oyama et al., 1980a; Spiess and Noe, 1985]. In catfish, a 22 residue form of SRIF (cSRIF-22) is derived from proSRIF-II; 7 of the 14 amino acids at the carboxyl terminus of cSRIF-22 are different from those found in SRIF-14 [Andrews et al., 1984b; Oyama et al., 1980a]. In the anglerfish, the major product of proSRIF-II appears to be a 28 amino acid molecule (aSRIF-28) in which 2 of the 14 amino acids at the carboxyl terminus are different from SRIF-14 [Andrews et al., 1984a; Morel et al., 1984a, 1984b; Spiess and Noe, 1985]. Interestingly, the peptide produced from proSRIF-II in both teleost species undergoes some form of additional processing: cSRIF-22 contains a glycosylated Thr residue at position 5 [Andrews et al., 1984b], and aSRIF-28 contains a hydroxylated Lys residue at position 23 [Andrews et al., 1984a; Spiess and Noe, 1985]. Several SRIF molecules, which appear to be products of two separate precursors, have also been isolated from the pancreas of the coho salmon [Plisetskaya et al., 1986]. The major form of SRIF in the salmon pancreas is a 25 amino acid peptide (sSRIF-25) almost identical in structure to aSRIF-28, and, therefore, is probably derived from a precursor corresponding to the proSRIF-II identified in other teleost species. SRIF-14 is also present in the salmon pancreas, and appears to be derived from a proSRIF-I-like precursor.

The actions of SRIF-14 and SRIF-28 on pituitary and pancreatic hormone release are well documented in mammalian species [Arimura, 1981;

Arimura and Culler, 1985]. In lower vertebrates, the role of SRIF in the regulation of hormone release has not been as extensively studied. The various teleostean SRIF molecules were isolated from pancreatic islet tissue, but only a limited number of studies are available to suggest that SRIF may influence pancreatic hormone release in teleosts [Epple et al., 1983; Ince, 1980; Plisetskaya et al., 1986; Ronner and Scarpa, 1982]. SRIF-14-like immunoreactivity has been detected in the brain and pituitary of several teleost species [Dubois et al., 1979; Kah et al., 1982; Olivereau et al., 1984a, 1984b; Vigh-Teichmann et al., 1983], and the close proximity of the immunoreactive SRIF-14 fibers to the somatotrophs in the teleost pituitary led to the suggestion of a functional relationship between the SRIF-14 fibers and the GH secreting cells [Dubois et al., 1979; Kah et al.; 1982; Olivereau et al., 1984b].

In the present study, the influence of SRIF-14 on GH release from the goldfish pituitary was examined in vitro using a pituitary perifusion system. Previous studies on the effects of SRIF on GH release in teleosts [Cook and Peter, 1984; Fryer et al., 1979; Rivas et al., 1986; Wigham and Batten, 1984] have examined the biological actions of SRIF-14 only, and the influence of other SRIF peptides on pituitary hormone release in teleosts is not known. Therefore, the effects of mammalian SRIF-28 and the teleost peptides cSRIF-22 and sSRIF-25 on GH secretion from the goldfish pituitary were also compared to the effects of SRIF-14. Results from this study provide fundamental information about the influence of the various SRIF peptides on GH secretion in a teleost species.

2.2 MATERIALS AND METHODS

Experimental Animals

Goldfish of the common or comet varieties were purchased from Grassyforks Fisheries (Martinsville, IN) or Ozark Fisheries (Stoutland, MO). The goldfish were maintained on a 16 hours light: 8 hours dark photoperiod in flow-through aquaria at 17 °C for a minimum of 4 weeks prior to the experiments. The fish were fed twice daily to satiation with commercially prepared fish food (Clark's New Age Fish Feed Pellets, Moore-Clark Co., LaConner, WA). For Experiments 1 and 2, goldfish were acquired in January; for Experiments 3 and 4, fish were obtained in May and February, respectively. Only female goldfish were used in the present study.

Pituitary Perifusion System

Experiments were conducted using a perifusion system adapted from procedures used to study gonadotropin (GTH) secretion from goldfish pituitary fragments [Chang et al., 1984; MacKenzie et al., 1984]. Pituitaries were rapidly removed from goldfish killed in excess anaesthetic (tricaine methanesulfonate; Sigma Chemical Co., St. Louis, MO). The whole pituitaries were placed in a petri dish containing approximately 10 ml of medium (Hank's basic salts solution diluted 20 % with distilled water and supplemented with 15 mM Hepes buffer and 0.1 % bovine serum albumin, HBSS [MacKenzie et al., 1984]). The petri dish containing the pituitaries was kept on wet ice until collection was completed. Following collection, the pituitaries were placed in a sterile controlled environment room maintained at 17 °C for all subsequent procedures.

The neurointermediate lobe was separated from the rest of the pituitary and discarded. The pars distalis was diced into fragments (less than 0.5 mm^2) using fine forceps and scissors. The fragments were then washed twice with HBSS and fragments equivalent to 4 to 5 pituitaries were placed between two layers of Cytodex carrier beads (Pharmacia, Dorval, Que.) in 0.5 ml perifusion chambers. A three-way valve was used to connect the chamber in each perifusion column to a reservoir of HBSS maintained at room temperature (17 °C); a second reservoir was also connected to the three-way valve and was used to administer the test solutions directly into the perifusion chamber. A peristaltic pump with an adjustable flow rate was positioned below the chamber and connected to the perifusion column with tubing.

For Experiments 1 and 2, the flow rate of HBSS through the columns was adjusted to 15 ml/hour and the pituitary fragments were allowed to equilibrate for a period of 2 hours. Preliminary experiments demonstrated that GH secretion from the fragments usually reached a basal level during this period. As a result of the length of Experiment 3, fragments were placed in the chambers on the previous evening and allowed to equilibrate overnight for a period of 8 to 10 hours. During this overnight equilibration period, the flow rate was adjusted to 5 ml/hour and the perifusion medium consisted of Medium 199 containing Hank's basic salts (Gibco Laboratories, Grand Island, NY) and supplemented with 15 mM Hepes buffer and Nystatin (56 U/ml). Two hours prior to the start of the experiment, the medium was switched to HBSS and the flow rate was adjusted to 15 ml/hour. Experiment 4 was also performed on pituitary fragments allowed to equilibrate overnight. The results obtained using the overnight incubation were comparable to results obtained using a 2 hour equilibration period at a similar time of the year. A constant basal rate of GH secretion was maintained throughout the experimental period using both of these protocols.

In Experiment 1, goldfish pituitary fragments in 3 perifusion columns were exposed to increasing concentrations of SRIF-14. The fragments were exposed to each concentration of SRIF-14 for 25 minutes, sequentially from the lowest to the highest concentration. In Experiment 2, pituitary fragments in 4 perifusion columns were exposed to 2 minute pulses of increasing concentrations of SRIF-14. Following each pulse of SRIF-14, the fragments were perifused for 63 minutes with HBSS. In Experiment 3, 2 minute pulses of SRIF-14 were alternated with a 2 minute pulse of an equivalent dose of either mammalian SRIF-28 (3 columns) or cSRIF-22 (4 columns). The concentrations of SRIF-14 tested in Experiment 3 were 0.1, 1, and 10 nM, whereas 0.1, 1, 10 and 100 nM dosages of the other SRIF peptides were tested. Following each pulse of test solution, the fragments were exposed to HBSS only for 63 minutes. In Experiment 4, the influence of a single concentration of SRIF-14 on GH secretion was compared to a corresponding dose of sSRIF-25 on fragments in individual perifusion columns. Pituitary fragments in 6 perifusion columns were exposed to a 2 minute pulse of one of three concentrations of SRIF-14: 0.1, 1, or 10 nM. Following exposure to SRIF-14, the fragments were perifused with HHBS for 58 minutes, and then exposed to a 2 minute pulse of sSRIF-25 at a concentration corresponding to the SRIF-14 dose.

During the experimental period, the perifusion medium was collected at 5 minute (1.25 ml) intervals using an automatic fraction collector. Fractions from up to 6 separate columns could be collected simultaneously. The GH content of each fraction from Experiments 1, 2 and 3 was determined using a carp GH radioimmunoassay (RIA) previously validated for measuring circulating levels of GH in the goldfish [Cook et al., 1983]. In this RIA, the displacement curves produced by serial dilutions of the perifusion samples are parallel to the carp GH standard, indicating that this RIA is also suitable for measuring GH released from goldfish pituitary fragments in vitro. The GH level in fractions of perifusate from Experiment 4 was measured using the carp GH RIA described in Appendix I.

Peptide Solutions

SRIF-14 and mammalian SRIF-28 were purchased from Sigma Chemical Co. and Penninsula Laboratories (Belmont, CA), respectively. The cSRIF-22 used in the present study was generously provided by Dr. P.C. Andrews (Department of Biochemistry, Purdue University, West Lafayette, IN 47907). The cSRIF-22 was purified from catfish pancreatic islets [Andrews et al., 1984b] and consisted of a mixture of the glycosylated forms cSRIF-22a and cSRIF-22b described previously [Andrews et al., 1984b]. sSRIF-25 purified from the pancreas of coho salmon [Plisetskaya et al., 1986] was generously provided by Dr. E.M. Plisetskaya (Department of Zoology, University of Washington, Seattle, WA 98195). The test peptides were dissolved in HBSS and administered directly to the fragments from a separate reservoir connected to the perifusion chamber by a three-way valve. Several concentrations of each test solution were made immediately prior to each experiment and kept at 4 °C until administration, at which time the temperature of the solution was allowed to equilibrate to 17 °C.

Calculation of Dose Response Curves

In Experiments 1 and 2, the fraction from each column with the highest GH value (ng GH/ml medium) was determined and the GH levels in all other fractions from that column were expressed as a percent of this value. This transformation to percent of the highest GH value in each column (% of maximum) allowed data from several columns to be combined for graphic presentation (Figures 2.1 and 2.3). To construct dose response curves for each peptide, the GH secretion rate (ng GH secreted/minute) following each dose of peptide was calculated and the response was expressed as a percentage of the basal secretion rate (Figures 2.2, 2.4 and 2.5; Table 2.1). In Experiment 1, the basal secretion rate was calculated as the average secretion rate during the first 40 minutes prior to exposure to SRIF-14; the rate of GH secretion during exposure to each concentration of SRIF-14 was determined as the average GH secretion rate during the 25 minute exposure period. In Experiments 2, 3 and 4, the secretion rate over the 25 minute period preceding each 2 minute pulse of peptide was determined; analysis of variance revealed no significant differences between the pre-pulse secretion rates within each column. Therefore, the basal secretion rate in each column was defined as the average secretion rate in all the pre-pulse periods. The response to each peptide solution was determined as the average GH secretion rate over the 25 minute period immediately following each pulse. This rate was expressed as a percent of the basal secretion rate. For Experiments 1, 2 and 3, data from several columns were combined, and dose response curves and parameters were analyzed using the ALLFIT computer program [De Lean et al., 1978]. In Experiment 3, the number of doses used for SRIF-14 was insufficient for analysis with this program, and consequently, only the response curve for SRIF-28 was analyzed with the ALLFIT program. Parallel line statistical analysis [Pekary, 1979] was used to compare the slope of the regression line calculated from the response to the 0.1 through 10 nM doses of SRIF-14 to the slopes of the regression lines calculated for similar doses of SRIF-28 and cSRIF-22. The dose reponse curves for SRIF-14 and sSRIF-25 from Experiment 4 were also compared using the parallel line statistical analysis [Pekary, 1979].

2.3 RESULTS

Experiment 1

In this experiment, continuous exposure to increasing levels of SRIF-14 resulted in a rapid and dose-dependent decrease in GH released from the pituitary fragments (Figure 2.1). Following removal of SRIF-14 from the medium (fractions 59 through 65), GH secretion rapidly returned to near basal levels (Figure 2.1). Analysis of the dose response curve for this experiment (Figure 2.2) indicates that the half maximal effective dose (ED_{50}) of SRIF-14 is 65 nM in this experiment.

Experiment 2

In this experiment, 2 minute pulses of increasing doses of SRIF-14 resulted in a rapid and dose-dependent decrease in the rate of GH secretion from the fragments (Figure 2.3). GH secretion after each pulse returned to levels close to the baseline, although a longer period of time was required to reach basal levels with the higher doses of SRIF-14. Analysis of the dose response curve for Experiment 2 (Figure 2.4) indicates that the ED_{50} for SRIF-14 is 1.3 nM in this experiment.

Experiment 3

Two minute pulses of SRIF-14 were alternated with 2 minute pulses of corresponding doses of either SRIF-28 or cSRIF-22. Analysis of the dose response curve from this experiment indicates that both SRIF-14 and SRIF-28 cause a dose-dependent decrease in GH secretion from goldfish pituitary fragments (Figure 2.5). However, cSRIF-22 at concentrations up to 100 nM was ineffective in altering GH secretion. Regression analysis revealed that the response of the fragments to SRIF-28 was identical to the response elicited by SRIF-14 over the range of concentrations tested in this experiment. The ED_{50} calculated for SRIF-28 in this experiment is 1.0 nM.

Experiment 4

Individual columns were exposed to a 2 minute pulse of SRIF-14 followed by exposure to an eqivalent dose of sSRIF-25. Profiles of GH in the perifusion medium from three representative columns from Experiment 4 are shown in Figure 2.6. Exposure to SRIF-14 resulted in a dose-dependent decrease in the secretion rate of GH, whereas sSRIF-25 was ineffective in altering GH release from goldfish pituitary fragments (Table 2.1).

2.4 DISCUSSION

This study demonstrates that SRIF-14 is a potent inhibitor of GH release from goldfish pituitary fragments *in vitro*, providing additional support for the hypothesis that SRIF-14 functions as a GH release-inhibitory factor in the goldfish. Previously, intraperitoneal injection of SRIF-14 was shown to decrease circulating levels of GH in the goldfish [Cook and Peter, 1984]. In addition, destruction of the nucleus preopticus periventricularis (NPP), a region of the goldfish forebrain containing abundant perikarya with SRIF-14 immunoreactivity [Kah *et al*., 1982; Olivereau *et al*., 1984a], results in increased GH cell secretory activity [Fryer, 1981], increased circulating levels of GH [Cook and Peter, 1983], and increased growth rates [Cook and Peter, 1983], presumably as a result of the destruction of a somatostatinergic projection from the NPP to the pituitary [Fryer, 1981; Cook and Peter, 1983]. The present study also confirms and extends the results of two studies in the tilapia (*Oreochromis* (*Sarotherodon*) *mossambicus*) [Fryer *et al*., 1979; Rivas *et al*., 1986] and a study in *Poecilia latipinna* [Wigham and Batten, 1984] in which SRIF-14 at concentrations as low as 30 nM were shown to inhibit GH release *in vitro*. However, results from the present study indicate that the pituitary of teleosts may actually be responsive to much lower concentrations of SRIF-14, as doses as low as 0.1 nM were effective in inhibiting GH release from the goldfish pituitary.

The ED$_{50}$ calculated for SRIF-14 following exposure of goldfish pituitary fragments to 2 minute pulses of SRIF-14 was 1.3 nM, very similar to the ED$_{50}$ for SRIF-14 in static cultures of rat pituitary cells [Brazeau *et al*., 1973; Vale *et al*., 1975] and to concentrations of SRIF-14 used in studies on perifused rat pituitary cells [Cowan *et al*., 1983]. SRIF-14 acts very rapidly to inhibit GH secretion from the goldfish pituitary; in Experiment 2, decreased levels of GH in the perifusion medium were observed in the first 5 minutes following each pulse of SRIF-14. Following exposure to pulses of SRIF-14, GH secretion returned to basal rates, although the length of time required to recover to baseline was increased at the higher doses of SRIF-14. The goldfish pituitary fragments did not show a rebound in GH secretion following removal of SRIF-14; in mammals, GH secretion rebounds to rates much higher than basal rates after exposure to SRIF-14 *in vitro* [Cowan *et al*., 1983].

Analysis of the dose response curves from Experiments 1 and 2 revealed some differences between the potency of SRIF-14 in the two experiments. This difference is probably related to the different protocols used in the two experiments: increasing doses of SRIF-14 were continuously applied to the fragments in Experiment 1, whereas in Experiment 2, GH secretion was allowed to return to the basal rate before application of the next dose of SRIF-14. The higher ED$_{50}$ in Experiment 1 indicates that the pituitary fragments were less responsive to SRIF-14 in this experiment, suggesting that prior exposure to SRIF-14 may influence the response of the goldfish pituitary to subsequent SRIF-14 exposure.

Although SRIF-14 at concentrations of 1 and 10 nM were effective in inhibiting GH secretion in Experiment 3, the magnitude of the response elicited by these doses of SRIF-14 was much less than that observed in Experiments 1, 2 or 4. A previous study [MacKenzie et al., 1984] reported that GTH secretion from goldfish pituitary fragments in response to GTH-releasing factors is influenced by the time of year and/or the reproductive condition of the experimental animals. In the present study, Experiments 1, 2 and 4 were conducted in early February, whereas the goldfish used in Experiment 3 were acquired in May, suggesting that the response of the pituitary to SRIF-14 may change seasonally in goldfish. Serum levels of GH have been shown to vary on a seasonal basis in the goldfish [Marchant and Peter, 1986], with the highest serum GH levels occurring in March through June, and lower levels occurring in the winter months. In the present study, the goldfish pituitary appears to be most capable of responding to SRIF-14 at a time of year when serum GH levels are lowest. Conversely, the magnitude of the response elicited by SRIF-14 was lower at the time of year when circulating GH levels are highest in the goldfish. The increased pituitary responsiveness to SRIF-14 in Experiments 1, 2 and 4 also corresponds to the time of year when the pituitary content of immunoreactive SRIF is the highest in the goldfish [Chapter 3].

In mammals, SRIF-28 and SRIF-14 are produced from the same proSRIF molecule [Goodman et al., 1982; Shen et al., 1982], and SRIF-28 has at least equal potency with SRIF-14 in inhibiting GH release in vivo and in vitro [Brazeau et al., 1981; Millar et al., 1983; Tannenbaum et al., 1982]. It is not known if the proSRIF-I molecule in teleosts is also processed to a peptide similar to SRIF-28 containing the sequence of SRIF-14 at its carboxyl terminus. However, results from Experiment 3 of the present study indicate that mammalian SRIF-28 is identical in potency to SRIF-14 in inhibiting GH release from goldfish pituitary fragments; in Experiment 3 the ED_{50} of SRIF-28 was calculated as 1.0 nM whereas the ED_{50} of SRIF-14 in Experiment 2 was 1.3 nM.

The presence of two distinct proSRIF molecules in teleosts has led to the suggestion that the peptides derived from the two precursors may have different physiological functions [Andrews and Dixon, 1981; Andrews *et al*., 1984b; Fletcher *et al*., 1983; Hobart *et al*., 1980; Magazin *et al*., 1982; Minth *et al*., 1982; Morel *et al*., 1984b; Plisetskaya *et al*., 1986; Spiess and Noe, 1985]. cSRIF-22 has been shown to inhibit GH release from rat pituitary cells [Andrews *et al*., 1984b; Oyama *et al*., 1981], but with only 0.01 to 0.1 % of the potency of SRIF-14. cSRIF-22 also displaces SRIF-14 from a rat pituitary membrane preparation, but once again with only a fraction of the potency of SRIF-14 [Oyama *et al*., 1981]. In the same study, cSRIF-22 was also found to inhibit insulin and glucagon release from the perfused rat pancreas, with approximately 10 % of the potency of SRIF-14. Thus, in the rat, cSRIF-22 does possess biological activities similar to SRIF-14, albiet with greatly reduced potency. The influence of sSRIF-25 on hormone release in mammals has not been studied, although Plisetskaya *et al*. [1986] have shown that sSRIF-25 decreases circulating insulin levels in coho salmon.

The present study is the first to examine the influences of cSRIF-22 and sSRIF-25 on pituitary hormone release in a teleost species. In this study, cSRIF-22 at doses up to 100 nM had no effect on GH release from goldfish pituitary fragments, whereas a 1 nM dose of SRIF-14 inhibited GH secretion from the same fragments. The apparent lack of activity of cSRIF-22 is not surprising as 7 of the 14 amino acids at the carboxyl terminus are different from those in SRIF-14 [Andrews *et al*., 1984b]. In mammalian systems, structure activity studies [Vale et al., 1975; Vale et al., 1977] have shown that alterations to several of the amino acids in SRIF-14, including Phe^{6}, Phe^{7}, Trp^{8}, Lys^{9} and Phe^{11}, result in a very reduced potency of the SRIF-14 molecule. Several of the corresponding residues in cSRIF-22 have been altered [Andrews et al., 1984b]. Alterations to these positions in cSRIF-22 as well as in other regions of the molecule may contribute to the lack of potency observed in the present study. cSRIF-22 is also glycosylated at Thr^{5}, a feature unique to this form of SRIF which may also influence its biological activity [Andrews et al., 1984b].

In a separate experiment, sSRIF-25 at concentrations up to 10 nM was also ineffective, compared to SRIF-14, in altering GH secretion from the goldfish pituitary. The lack of any effect of sSRIF-25 in this experiment is somewhat surprising as only 2 of the 14 amino acids at the carboxyl terminus of sSRIF-25 are different from those in SRIF-14, with the remainder of the molecule also having a fairly high degree of homology with mammalian SRIF-28 [Plisetskaya et al., 1986]. However, sSRIF-25 is almost identical to aSRIF-28, and Speiss and Noe [1985] previously suggested that aSRIF-28 would not be a potent inhibitor of GH release, as the 2 substitutions in the carboxyl region of aSRIF-28 occur in the region of the molecule thought to be important for biological activity in mammalian species. Morel et al. [1984b] demonstrated that aSRIF-28 at concentrations ranging from 0.1 to 10 nM inhibited GH secretion from the rat anterior pituitary gland, although with reduced potency compared to SRIF-14. Therefore, the results of the present study support the prediction of Spiess and Noe [1985], and indicate that very slight alterations in the SRIF molecule, especially in the carboxyl region, result in the loss of biological activity in terms of inhibition of GH secretion from the goldfish pituitary.

The presence of the identical SRIF-14 molecule in species from a wide phylogenetic range of vertebrates [Andrews and Dixon, 1981; Brazeau *et al*., 1973; Conlon *et al*., 1985; Noe *et al*., 1979; Plisetskaya *et al*., 1986; Spiess *et al*., 1979] indicates strong evolutionary pressures to maintain the integrity of the SRIF-14 molecule throughout vertebrate evolution, presumably because this molecule fulfills a biological function necessary for the survival of vertebrates. Results from the present study provide support for the hypothesis that SRIF-14 is an important regulator of GH secretion in the goldfish, demonstrating that at least one biological action of SRIF-14 has been as highly conserved as its structure throughout vertebrate evolution.

Figure 2.1 has been published elsewhere (see Marchant _et al._, 1987. Regulatory Peptides _17_: 41-52), and has been removed because of the unavailability of copyright permission. This figure demonstrates the inhibition of GH secretion from perifused fragments of the goldfish pituitary following sequential exposure to increasing concentrations of SRIF-14.

Figure 2.2 has been published elsewhere (see Marchant _et_ _al_., 1987. Regulatory Peptides _17_: 41-52), and has been removed because of the unavailability of copyright permission. This figure demonstrates the dose-dependent inhibition of GH secretion from perifused fragments of the goldfish pituitary gland by sequential exposure to increasing concentrations of SRIF-14.

Figure 2.3 has been published elsewhere (see Marchant et al., 1987. Regulatory Peptides 17: 41-52), and has been removed because of the unavailability of copyright permission. This figure demonstrates the inhibition of GH secretion from perifused fragments of the goldfish pituitary following exposure to two minute pulses of various concentrations of SRIF-14.

Figure 2.4 has been published elsewhere (see Marchant _et al._, 1987. Regulatory Peptides _17_: 41-52), and has been removed because of the unavailability of copyright permission. This figure demonstrates the dose-dependent inhibition of GH secretion from perifused fragments of the goldfish pituitary gland following exposure to two minute pulses of various concentrations of SRIF-14.

Figure 2.5 has been published elsewhere (see Marchant *et al.*, 1987. Regulatory Peptides *17*: 41-52), and has been removed because of the unavailability of copyright permission. This figure demonstrates the dose-dependent inhibition of GH secretion from perifused fragments of the goldfish pituitary gland following exposure to SRIF-14 and SRIF-28, but not cSRIF-22.

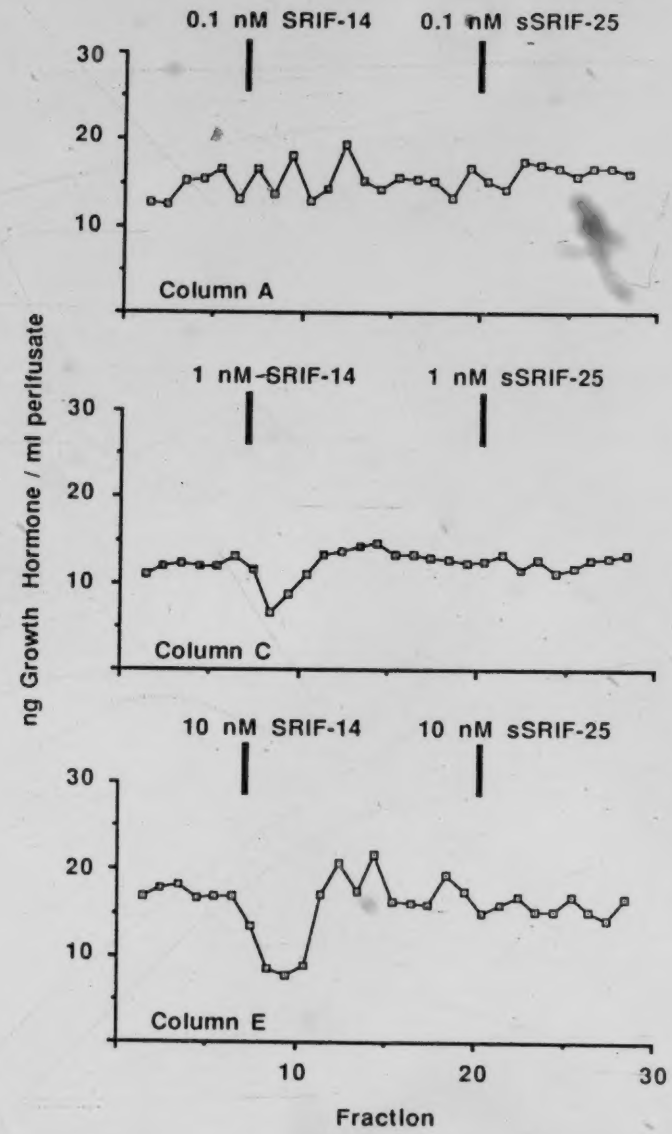

Figure 2.6. Effect of 2 minute pulses (black bars) of three doses of SRIF-14 followed by a corresponding dose of sSRIF-25 on GH levels in medium collected at 5 minute (1.25 ml) intervals from three individual perifusion columns containing fragments of the goldfish pituitary.

Table 2.1. Growth hormone secretion rates (% of basal) in individual pituitary perifusion columns following exposure to 2 minute pulses of equivalent concentrations of SRIF-14 and sSRIF-25 at 60 minute intervals.

		GH Secretion Rate (% of basal)	
Column	nM Peptide	SRIF-14	sSRIF-25
A	0.1	100	109
B	0.1	97.6	108
C	1.0	79.1	97.5
D	1.0	83.4	95.9
E	10	63.4	94.5
F	10	68.1	95.7

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3. THE RELATIONSHIP BETWEEN SERUM GROWTH HORMONE LEVELS AND THE BRAIN AND PITUITARY CONTENT OF IMMUNOREACTIVE SOMATOSTATIN IN THE GOLDFISH

3.1 INTRODUCTION

Immunocytochemical studies in a wide variety of teleost species, including the goldfish, have shown that cell bodies and nerve fibers containing immunoreactive somatostatin (irSRIF) are widely distributed throughout the central nervous system [Dubois *et al.*, 1979; Grau *et al.*, 1985; Kah *et al.*, 1982; Olivereau *et al.*, 1984a, 1984b; Vigh-Teichman *et al.*, 1983]. The regional distribution of irSRIF in the brain appears to be quite similar in all of the teleost species studied. Immunoreactive cell bodies are present in several nuclei in the preoptic and periventricular regions, including the nucleus preopticus (NPO), nucleus preopticus periventricularis (NPP), nucleus anterioris periventricularis (NAPv), and the nucleus lateralis tuberis (NLT). Fibers from nuclei in the forebrain form a dense plexus of irSRIF fibers in the periventricular region of the hypothalamus. At least some immunoreactive fibers from this plexus course through the basal hypothalamus and the infundibulum to terminate within the pituitary gland. In cyprinid species such as the goldfish [Kah *et al.*, 1982; Olivereau *et al.*, 1984b] irSRIF fibers penetrate into the proximal pars distalis and are found scattered among the somatotrophs. In other species such as the eel (*Anguilla anguilla*) [Olivereau *et al.*, 1984a] and various salmonids [Dubois *et al.*, 1979; Olivereau *et al.*, 1984a], irSRIF fibers terminate on the basal lamina of the neurohypophyseal tissue within the proximal pars distalis. The tilapia (*Oreochromis mossambicus*) is unique among the teleost species studied to date in that irSRIF fibers are also found in neurohypophyseal tissue within the rostral pars distalis [Grau *et al*., 1985].

The close association of the irSRIF fibers with the somatotrophs in the teleost pituitary provides an anatomical basis for a functional relationship between SRIF and growth hormone (GH) secretion in teleosts. Extensive evidence has accumulated to support the hypothesis that hypothalmic SRIF functions as a GH release-inhibitory factor in mammals [Arimura and Culler, 1985]. SRIF has been shown to decrease circulating levels of GH in the goldfish [Cook and Peter, 1984], and to inhibit GH secretion *in vitro* from the pituitary of goldfish [see Chapter 2], tilapia [Fryer *et al*., 1979; Rivas *et al*., 1986] and *Poecilia latipinna* [Wigham and Batten, 1984], suggesting that SRIF also functions as a GH release-inhibitory factor in teleosts. Two brain lesion studies in the goldfish [Cook and Peter, 1983; Fryer, 1981] provided evidence suggesting that the preoptic area of the forebrain exerts an inhibitory influence on GH secretion, and it was hypothesized that this inhibitory projection may be somatostatinergic [Cook and Peter, 1983; Fryer, 1981]. In another study [Olivereau *et al*., 1984a], a decrease in the amount of irSRIF detected by immunocytochemistry in the pituitary was associated with increased secretory activity of the somatotrophs, following long-term starvation in the eel and carp. Although these studies suggest that SRIF in the brain and pituitary may be involved in the neuroendocrine regulation of GH secretion in teleosts, direct evidence linking changes in the brain and pituitary content of irSRIF to variations in circulating GH levels in a teleost species is not currently available.

In the present study, the relationship between endogenous brain and pituitary SRIF and circulating GH levels was studied in the goldfish using two experimental approaches. First, the amount of irSRIF in the pituitary and various brain regions was measured by radioimmunoassay (RIA) at several times throughout the year, in relation to seasonal changes in serum GH levels [Marchant and Peter, 1986]. Second, the brain origin of irSRIF fibers in the goldfish pituitary was studied by measuring the amount of irSRIF in the pituitary following destruction of specific brain areas by radiofrequency lesions.

3.2 MATERIALS AND METHODS

Experimental Animals

Goldfish of the common or comet varieties were purchased from Grassyforks Fisheries (Martinsville, IN). For the seasonal experiments, goldfish were obtained at five different times throughout the year; female goldfish (15 to 25 g) were selected and placed in 96 L flow-through aquaria. The fish were maintained for 4 weeks at photoperiods and temperatures simulating natural (Edmonton) environmental conditions appropriate for the time of year when the experiment was conducted. Photoperiods were adjusted weekly to account for natural changes in daylength; natural dawn and dusk conditions were also simulated. The temperatures and photoperiods used in the present study were similar to those used previously to examine seasonal changes in somatic growth and serum GH levels in the goldfish [Marchant and Peter, 1986]. For the brain lesion experiments, female goldfish were placed in 96 L flow-through aquaria and maintained on a photoperiod of 16 hours light:8 hours dark (16L:8D) at 18 °C for a minimum of three weeks before each experiment. In all experiments, the fish were fed to satiation twice daily with commercially prepared fish food (Clark's New Age Fish Feed Pellets, Moore-Clark Co., LaConner, WA).

Seasonal Experiments

Groups of goldfish were sacrificed following acclimation to simulated natural photoperiods and temperatures at five separate times throughout the year: early February, early May, mid-June, late July, and late November. The goldfish were placed in anaesthetic (0.05% tricaine methanesulphonate; Sigma Chemical Co., St. Louis, MO) until opercular movements ceased. Blood samples were taken from the caudal vasculature using procedures described previously [Marchant and Peter, 1986]. The blood samples were allowed to clot at 4 °C for several hours, centrifuged, and the serum collected and stored at -25 °C. Following blood sampling, the fish were killed by spinal transection. The cranial cavity was opened dorsally and the whole brain was removed by grasping the spinal cord with blunt forceps and cutting the cranial nerves with fine scissors as the brain was gently lifted dorsally. The brain was placed in a Petri dish over wet ice. The pituitary gland, which remained in the sella turcica following brain removal, was removed and placed in the Petri dish over wet ice. The brain was cut using fine scissors into various portions designated as the hypothalamus, telencephalon, thalamus+mid-brain, and the cerebellum+medulla (Figure 3.1).

SRIF in the brain and pituitary samples was extracted using procedures modified from those of King and Millar [1979]. Following separation, each brain region and pituitary was placed in a test tube containing 1.5 ml of ice cold 2 M acetic acid. The tissue samples were kept ice cold and homogenized for 30 seconds using a Polytron homogenizer. The homogenized sample was transferred to a second test tube and the test tube used for homogenization rinsed with 1.0 ml of ice cold acetic acid; this rinse was added to the homogenized sample. The samples were then centrifuged at 10,000g for 45 min at 4 °C. The supernatant from each sample was collected, an aliquot removed for protein measurement and the balance frozen on dry ice for irSRIF measurement. Tissue samples used for irSRIF measurement were lyophilized and stored at -25 °C until reconstitution in the assay buffer. The efficiency of this extraction procedure was estimated by adding 1000 pg of SRIF-14 to samples of the pituitary and each brain region prior to homogenization and extraction. Recovery of the added SRIF in samples from each area was greater than 87 %.

Brain Lesioning Experiments

Lesions were placed in the goldfish forebrain using the techniques detailed by Peter and Gill [1975]. The lesions were made using a radiofrequency lesion generator (Radionics Laboratories, Burlington, MA), coupled to a temperature-sensing electrode, to supply sufficient current to generate lesion temperatures of 80 to 90 °C for 30 seconds. In Experiment 1, large medial lesions were placed at the level of the NPP and NPO in the preoptic region (coordinates: A +1.2-1.4, M, D 1.9-2.0). In Experiment 2, large medial lesions were placed at the level of the NLT pars anterioris (NLTa) in the anterior-ventral hypothalamus (A +0.9, M, D 2.9), and more posteriorly (A +0.5, M, D 3.1) at the level of the NLT pars posterioris (NLTp). In sham-operated animals, the electrode was lowered into the brain at the same coordinates, but current was not applied.

In Experiment 1, the fish were sacrificed at 30 days following the operation, whereas in Experiment 2, the fish were sacrificed 8 days post-operation. At the time of sacrifice, the fish were placed in excess anaesthetic, blood sampled, and the pituitary removed and placed in 1.5 ml of ice cold 2 M acetic acid. SRIF in the pituitary was extracted as described for the seasonal experiments. Brains were also removed at the time of sacrifice, fixed in Bouin's solution, and the placement of the electrode was checked using routine histological procedures [Peter and Gill, 1975].

Hormone and Protein Measurement

The amount of GH in serum samples from the February, May, June and November sample times in the sesaonal study were measured using the carp GH RIA described previously [Cook et al., 1983]. The serum samples from fish sampled in July were inadvertently destroyed before measurement in the GH RIA. Serum GH levels in samples from Experiment 1 of the lesion study were also measured using this RIA [Cook et al., 1983], whereas GH levels in serum samples from lesion Experiment 2 were measured using the newly developed carp GH RIA [Appendix 1]. IrSRIF in brain and pituitary extracts was measured using the SRIF RIA described in Appendix 2. Prior to the SRIF RIA, each lyophilized tissue sample was reconstituted in 1.00 ml of the assay buffer. The reconstituted samples were centrifuged at 10,000g for 30 min at 4 °C, and the supernatant from each sample collected and stored at -25 °C until assayed for irSRIF. All samples were assayed in duplicate.

The protein content of the tissue extracts was determined using a commercially available protein assay kit (Biorad Laboratories, Mississauga, Ont.). Bovine serum albumin dissolved in 2 M acetic acid was used as the standard in the protein assay. The amount of protein in the pituitary samples was undetectable using this method; consequently, only the protein content in the four brain regions was determined.

Statistical Analysis

The irSRIF content in the four brain regions was calculated as the total amount of irSRIF in each area divided by the amount of protein in the supernatant collected after the initial centrifugation. irSRIF in the pituitary extracts was calculated as the total amount of irSRIF per pituitary. Differences between each time of the year in the amount of irSRIF in each region were analyzed by analysis of variance ($p<0.05$) followed by Duncan's multiple range test ($p=0.05$) [Steel and Torrie, 1960]. Serum GH data from the seasonal experiments were normalized using a logarithmic transformation, and analyzed using analysis of variance ($p<0.05$) followed by Duncan's multiple range test ($p=0.05$). For the brain lesion experiments, pituitary irSRIF content in the lesion and sham-operated groups was compared using Student's t-test ($p<0.05$). Serum GH data were normalized using a logarithmic transformation and compared using Student's t-test when variances were homogeneous (Experiment 1). The Kruskall-Wallis test ($p<0.05$) was used to compare serum GH levels when the variances were heterogeneous among treatment groups (Experiment 2).

3.3 RESULTS

Seasonal Experiments

Serum GH levels were significantly different between all of the groups of goldfish sampled at the various times of the year (Table 3.1). The highest serum GH levels were found in the group sampled in June, whereas the lowest levels were observed in the group sampled in November. In February and March, serum GH levels were intermediate between those measured in fish sampled in June and November. The seasonal profile in serum GH levels in the present study is very similar to seasonal changes in circulating GH levels reported previously in the goldfish [Marchant and Peter, 1986].

The amounts of irSRIF in the pituitary gland in fish sampled in February and November (Table 3.1) were similar, but significantly higher than the pituitary irSRIF content measured in fish from May, June and July. The pituitary irSRIF content was similar in groups of fish sampled in May, June and July.

The amount of irSRIF in the hypothalamus of fish sampled in November was significantly higher than in groups sampled at other times of the year (Table 3.1). In fish sampled in February, the hypothalamic irSRIF content was higher than in fish sampled in June, but similar to the hypothalamic irSRIF content found in fish sampled in May and July. The hypothalamic irSRIF content was similar in fish sampled in May, June and July.

The amount of irSRIF in the telencephalon of fish sampled in November was significantly higher than in fish sampled at other times of the year (Table 3.1). In February, the amount of irSRIF in the telencephalon was significantly higher than in groups of fish sampled in May, June and July. In May, the telencephalic content of irSRIF was significantly higher than in the group sampled in July. In June, the amount of irSRIF in the telencephalon was similar to that of fish sampled in July and May.

In November, the amount of irSRIF in the thalamus+mid-brain was significantly higher than in groups sampled at the other times of the year (Table 3.1). The irSRIF content of the thalamus+mid-brain in fish sampled in May was significantly higher than in groups sampled in February, June or July. The irSRIF content in the thalamus+mid-brain was similar in the groups of fish sampled in February, June and July.

The amounts of irSRIF measured in the cerebellum+medulla in July and May were similar, but significantly higher than in fish sampled in February, June and November (Table 3.1). In May, the irSRIF content of the cerebellum+medulla region was similar to that found in fish sampled in November, but significantly higher than that found in fish sampled in February and June. The irSRIF content in the cerebellum+medulla region in fish sampled in November was significantly higher than in fish sampled in February, but similar to the amount found in fish sampled in June. The irSRIF content of the cerebellum+medulla was similar in fish sampled in February and June.

Brain Lesion Experiments

The placement of the lesions was determined using the anatomical atlas of the goldfish forebrain described by Peter and Gill [1975]. In Experiment 1, the lesions were centered in the NPP and NPO, completely destroying these nuclei as well as areas lateral to the NPP and NPO (Figure 3.2). No damage to this area was evident in the brains of the sham-operated animals. At 30 days following the operation, serum GH levels were significantly higher in the group with lesions in the preoptic area compared to the sham-operated control group (Figure 3.3). The pituitary ir SRIF was signficantly lower in the group with preoptic lesions compared to the sham-operated group (Figure 3.3).

In Experiment 2, lesions placed in the anterior hypothalamus were centered around the NLTa, completely destroying this nucleus, the NLT pars lateralis, the anterior portion of the nucleus anterior tuberis (NAT),and the immediate surrounding area, but leaving posterior and lateral areas intact (Figure 3.4). Lesions placed more posteriorly in the basal hypothalamus were centered in the NLTp and completely destroyed this nucleus, the NLTa, NLT pars inferioris, the anterior portion of the NAT and in some cases, portions of the nucleus recessus lateralis (NRL), as well as most of the regions lateral to these nuclei (Figure 3.4); the lesions centered around the NLTp were located so as to destroy the majority of the fibers innervating the pituitary. No damage was evident in any of the brains from sham-operated fish. At 8 days following the operation, serum GH levels in fish with lesions centered in the NLTa were similar to the sham-operated controls (Figure 3.5). The pituitary irSRIF content in animals with these lesions was also similar to the sham-operated group (Figure 3.5). However, in fish with lesions centered in the NLTp, the pituitary irSRIF content was significantly lower compared to sham-operated fish (Figure 3.5). Serum GH levels in fish with the lesions centered in the NLTp tended to be slightly higher compared to the sham-operated group, although this difference was not statistically significant (Figure 3.5).

3.4 DISCUSSION

irSRIF was detected in all of the regions of the goldfish brain examined in the present study, including the pituitary gland. In goldfish, numerous irSRIF fibers are found within the proximal pars distalis, in close association with the somatotrophs [Kah et al., 1982; Olvereau et al., 1984a]. On this basis, the irSRIF detected in the pituitary gland in the present study is presumed to be contained within neuronal fibers in the pituitary. The irSRIF content of the telencephalic and hypothalamic regions of the goldfish brain was much higher than in the thalamus+mid-brain and cerebellum+medulla areas. Immunocytochemical studies have found irSRIF widely distributed in fibers and cell bodies throughout the brain of the goldfish and other teleost species [Kah et al., 1982; Vigh-Teichmann et al., 1983; Olivereau et al., 1984a, 1984b]. However, the majority of irSRIF fibers and cell bodies in the goldfish forebrain appear to be localized in the periventricular region of the preoptic area. Therefore, the larger amounts of irSRIF measured by RIA in the telencephalon and hypothalamus in the present study correspond to those regions previously demonstrated to contain the highest density of irSRIF perikarya and fibers.

In the present study, the amount of irSRIF in the pituitary gland of the goldfish was found to vary on a seasonal basis. The greatest amount of irSRIF in the pituitary was found in fish sampled in November and February, whereas the pituitary content of irSRIF in May, June and July was uniformly low. These results suggest that the pituitary content is highest at the time of year when serum GH levels are lowest and, conversely, lowest at the time of year when serum GH levels are the highest, providing additional support for a functional relationship between irSRIF contained in neuronal fibers within the proximal pars distalis and GH secretion. Interestingly, goldfish pituitary fragments in vitro were found to be most responsive to SRIF in January and February [Chapter 2], a time of year corresponding to a high pituitary SRIF content and low serum GH levels.

The amount of irSRIF present in the telencephalon and hypothalamus of the goldfish was also found to vary on a seasonal basis. These changes in irSRIF content were also inversely related to serum GH levels. In the telencephalon and hypothalamus, the irSRIF content was highest in November and February, the times of the year when circulating GH levels were the lowest. Conversely, in May, June and July, the irSRIF content of these regions decreased; serum GH levels are the highest at this time of year. Some differences in the amount of irSRIF present in the two other brain areas (the thalamus+mid-brain and the cerebellum+medulla) were observed at certain times of the year, but these differences do not appear to be related to seasonal changes in serum GH levels. Presumably, the irSRIF found in these latter brain regions has a function not necessarily related to pituitary hormone secretion. In mammals, SRIF is known to have various actions as a neurotransmitter or neuromodulator in the central nervous system [Arimura, 1981], and it is possible that SRIF may also have similar actions in the teleost brain [Olivereau et al., 1984a; Vigh-Teichman et al., 1983].

Previous studies in teleosts have suggested that SRIF present in the forebrain may have a role in the regulation of GH secretion.

Immunocytochemical studies have identified fibers containing irSRIF, originating from several nuclei in the periventricular area of the preoptic region, that form a dense plexus in this periventricular region, and appear to contribute to a preoptic-hypophyseal tract of irSRIF fibers reaching the infundibulum [Kah et al., 1982; Vigh-Teichmann et al., 1983; Olivereau et al., 1984a, 1984b]. Although the dissection of the brain in the present study did not allow measurement of the irSRIF content of individual nuclei in the goldfish forebrain, results of the present study indicate that gross changes in the irSRIF content of the goldfish forebrain can be related to seasonal changes in serum GH levels. This provides the possibility that changes in irSRIF in the telencephalon and hypothalamus may be involved in the neuroendocrine regulation of serum GH levels throughout the year.

Destruction of the NPP and NPO, two nuclei containing abundant irSRIF cell bodies, results in increased somatotroph secretory activity [Fryer, 1981], and increased serum GH levels and growth rates [Cook and Peter, 1983] in the goldfish, presumably as a result of the destruction of an inhibitory projection to the pituitary. Fryer [1981] also found that the number of peptidergic neurosecretory fibers innervating the proximal pars distalis was reduced following lesioning in the preoptic area, and suggested that the NPP-NPO region was the origin of peptidergic fibers inhibiting GH secretion from the pituitary of the goldfish. The present study also provides evidence that the preoptic region exerts an inhibitory influence on GH secretion in the goldfish, as serum GH levels were significantly increased in fish with the NPP-NPO lesion. Importantly, the finding that the amount of irSRIF in the pituitary is significantly reduced in animals with the NPP-NPO lesion, provides evidence directly supporting the hypothesis that the inhibitory projection from the preoptic area may be somatostatinergic.

Interestingly, the NPP-NPO lesions in the present study did not completely abolish irSRIF in the pituitary, suggesting that destruction of the NPP-NPO did not destroy all of the irSRIF projections to the pituitary. It is unlikely that the NPP or NPO together are the origin of all of the remaining irSRIF in the pituitary as these two nuclei were completely destroyed by the majority of the preoptic lesions. The animals were also sacrificed 30 days following the operation, allowing sufficient time for the degeneration of pituitary nerve fibers following destruction of irSRIF-containing neurons in the preoptic nuclei [Fryer, 1981]. The most likely explanation for the presence of irSRIF in the pituitary of goldfish with lesions in the preoptic area is that at least one other brain area also sends a projection of irSRIF to the pituitary. Immunocytochemical studies have identified irSRIF neurons in several other forebrain nuclei, including the NAPv and NLT, and it is possible that these nulcei may also be the origin of irSRIF fibers in the goldfish pituitary.

Lesions centered in the NLTa which spared lateral and posterior hypothalamic areas (Experiment 2) did not influence serum GH levels or the pituitary content of irSRIF. This indicates that irSRIF fibers innervating the goldfish pituitary probably do not originate or pass through the areas of the hypothalamus destroyed by the lesion. In contrast, lesions centered in the NLTp resulted in a dramatic reduction in the amount of irSRIF in the pituitary. These lesions were located so as to destroy a large region of the basal hypothalamus, and the very low levels of irSRIF in the pituitary suggests that the majority of irSRIF fibers innervating the pituitary were also destroyed. Thus, the majority of irSRIF projections to the goldfish pituitary appear to course through areas posterior and/or lateral to the NLTa. It is possible that nuclei containing irSRIF perikarya [Kah *et al.*, 1982; Olivereau *et al.*, 1984a] destroyed by the lesion in the basal hypothalamus may also be the origin of at least some of the irSRIF fibers in the pituitary.

Although the pituitary content of irSRIF was very low in fish with a lesion centered in the NLTp, serum GH levels were not signficantly increased in these animals. GH secretion in the goldfish [Cook and Peter, 1983; Peter and Fryer, 1983] and other teleosts [Ball, 1981] appears to be regulated at least in part by a stimulatory influence from the hypothalamus. In the present study, the lesions centered in the NLTp were located so as to destroy the majority of fibers innervating the pituitary; presumably, fibers containing subtances stimulatory to GH secretion would also have been destroyed by these lesions. Therefore, the absence of changes in serum GH levels in the presence of low amounts of irSRIF in the pituitary may be due to concommitant destruction of both stimulatory and inhibitory projections to the goldfish pituitary.

The present study is the first to examine the relationship between circulating levels of GH and the content of endogenous brain and pituitary irSRIF in a lower vertebrate. The seasonal experiments indicate that the amount of irSRIF present throughout the year in the pituitary and the goldfish forebrain is inversely related to seasonal variations in serum GH levels in the goldfish. The brain lesioning experiments provide evidence that nuclei in the preoptic region of the forebrain are the origin of at least some of the irSRIF nerve fibers in the goldfish pituitary. Together, these results provide additional support for a functional relationship between endogenous SRIF and GH secretion in the goldfish, and for the hypothesis that SRIF functions as a GH release-inhibitory factor in teleosts.

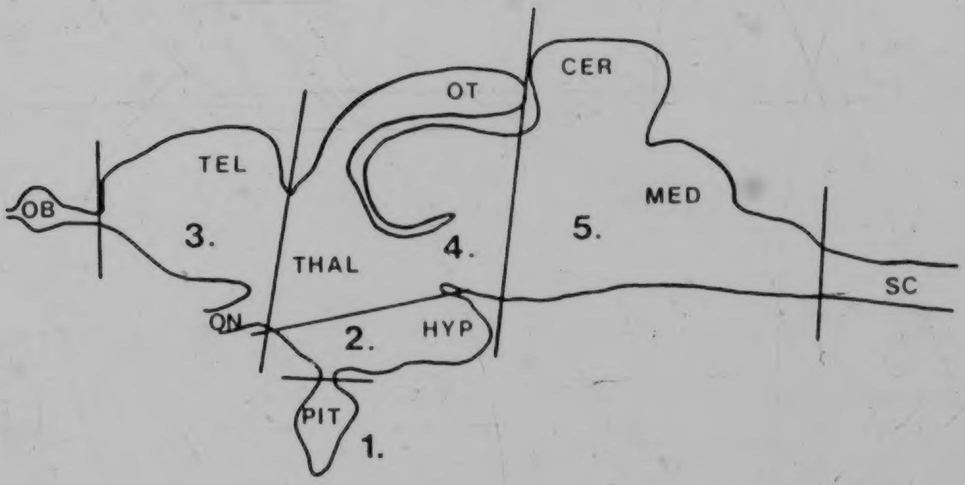


Figure 3.1. Planes of cuts used to dissect the goldfish brain into different regions for determination of irSRIF content. Designations as follows: 1 - pituitary; 2 - hypothalamus; 3 - telencephalon (including the preoptic area); 4 - thalamus+mid-brain; 5 - cerebellum+medulla. Abbreviations: OB - olfactory bulb; TEL - telencephalon; ON - optic nerve; OT - optic tectum; THAL - thalamus; HYP - hypothalamus; CER - cerebellum; MED - medulla oblongata; SC - spinal cord; PIT - pituitary.

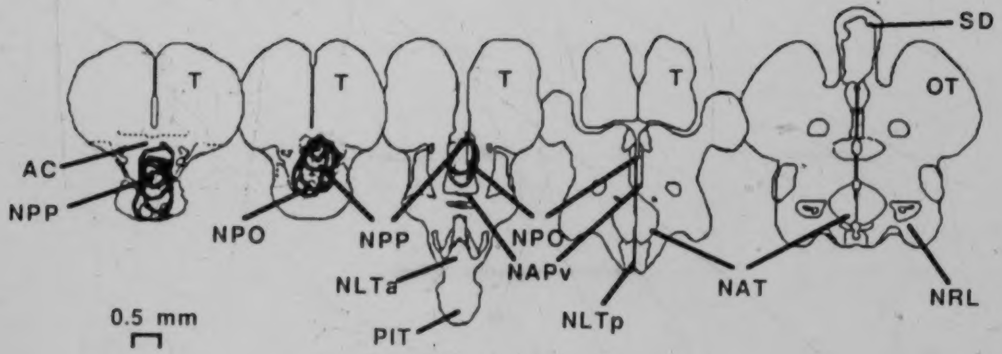


Figure 3.2. Diagrammatic summary of the placement of lesions (n=14) in the preoptic region of the goldfish forebrain (Experiment 1). The distance between the cross-sections is 0.3 mm. Abbreviations: T - telencephalon; NPP - nucleus preopticus periventricularis; OC - optic chiasma; AC - anterior commissure; NPO - nucleus preopticus; NAPv - nucleus anterioris periventriculairis; NLTa - nucleus lateral tuberis pars anterioris; NAT - nucleus anterior tuberis; NLTp - nucleus lateral tuberis pars posterioris; NRL - nucleus recessus lateralis; OT - optic tectum; SD - saccus dorsalis; PIT - pituitary.

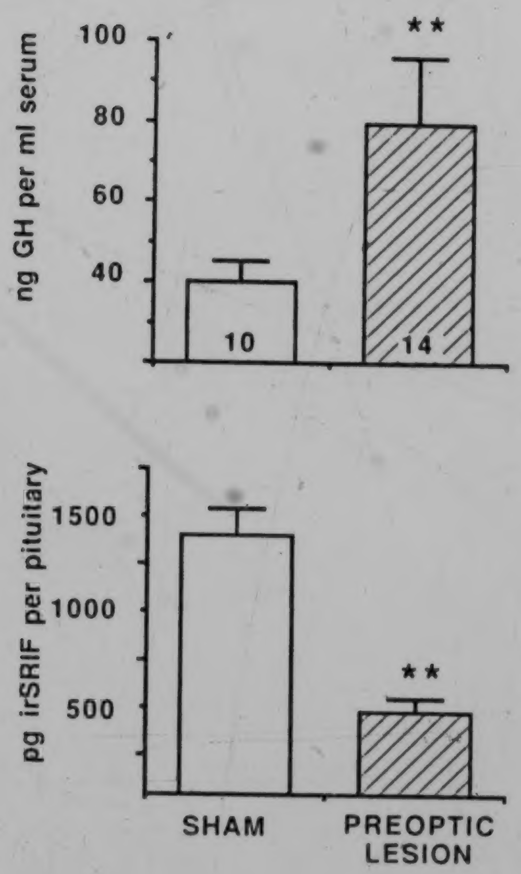
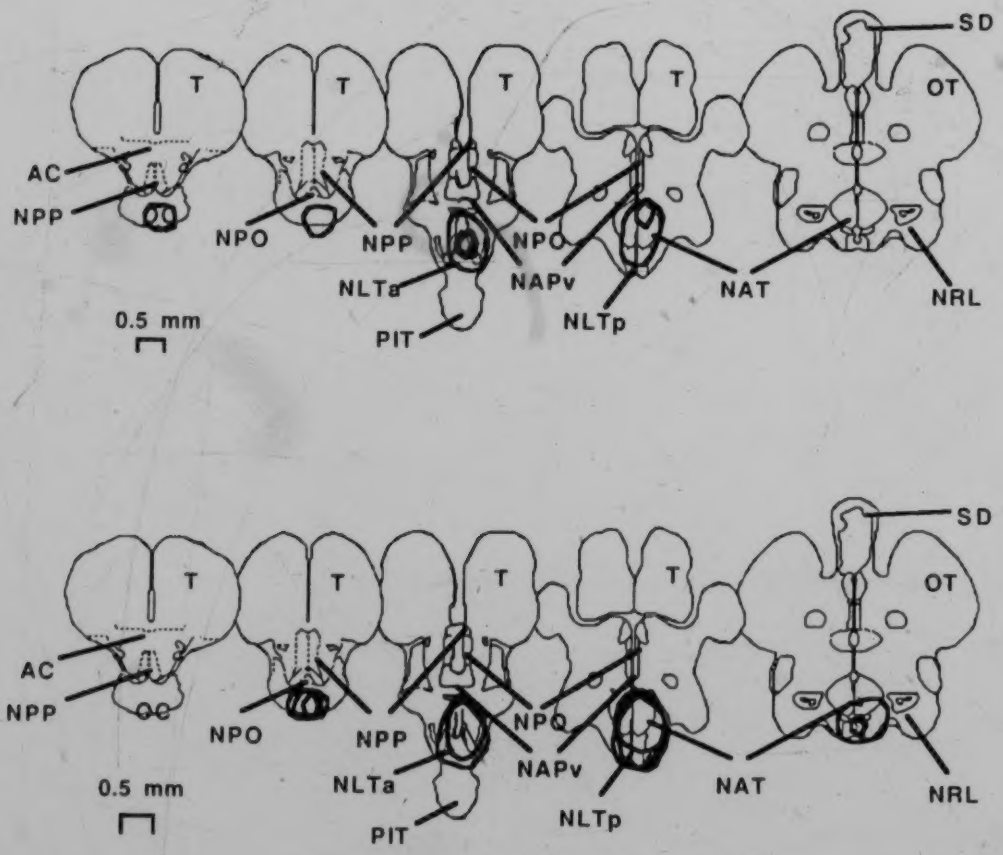


Figure 3.3. Serum growth hormone (GH) levels (top panel) and pituitary content of immunoreactive somatostatin (irSRIF; bottom panel) in female goldfish 30 days after sham operation or placement (Figure 3.2) of a lesion in the preoptic region of the goldfish. Sample sizes in each group are indicated along the abscissa in the top panel. Significant ($p<0.01$) differences in serum GH levels and pituitary irSRIF between the groups are indicated by asteriks.


SD
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NPO
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NRL
PIT
0.5 mm
SD
T
T
T
T
OT
AC
NPP
NPO
NPP
NPO
NLTa
NAPv
NLTp
NAT
NRL
PIT
0.5 mm

Figure 3.4. Diagrammatic summary of placement of lesions at the level of the NLTa in the anterior hypothalamus (top panel) and at the level of the NLTp in the basal hypothalamus (bottom panel) in female goldfish (Experiment 2). See Figure 3.2 for definition of the abbreviations .

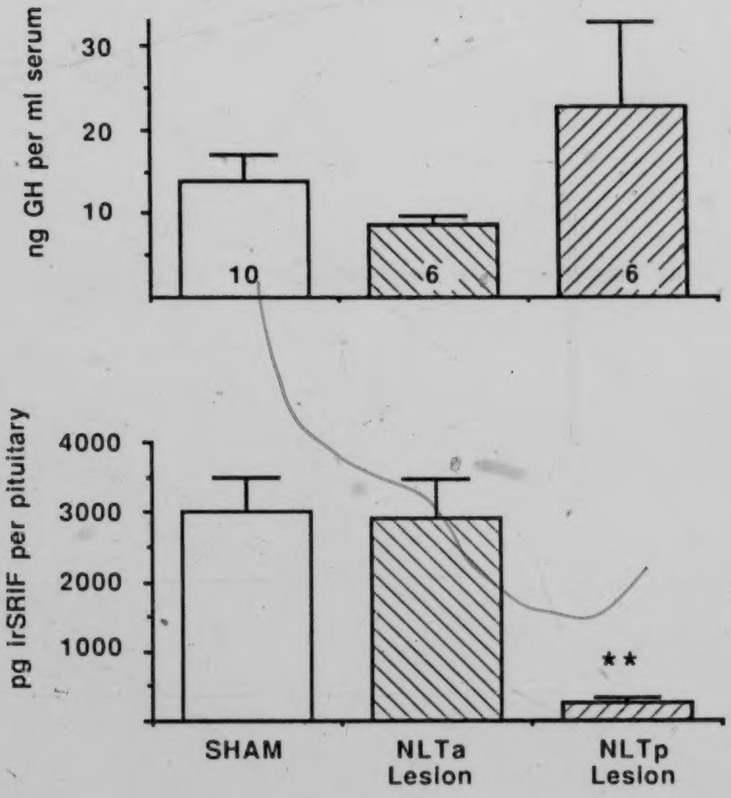

Figure 3.5. Serum growth hormone (GH) levels (top panel) and the content of immunoreactive somatostatin (irSRIF) in the pituitary (bottom panel) of female goldfish sacrificed 8 days following sham-operation or placement (Figure 3.4) of lesions centered in the NLTa or NLTp. Significant ($p<0.01$) differences in serum GH levels and the pituitary irSRIF content between sham-operated and the lesioned groups are indicated by asteriks. Sample sizes in each group are indicated along the abscissa in the top panel.

Table 3.1. Serum growth hormone (GH) levels and the content of immunoreactive somatostatin (irSRIF) in extracts of the pituitary and various brain regions in groups (n=30 to 40) of female goldfish sacrificed several times throughout the year after acclimation to simulated natural photoperiods and temperatures. Significant differences ($p < .05$) in serum GH levels or tissue irSRIF content between the various times of the year are indicated by superscript letters; groups with common superscripts are not significantly different.

	Time of Year				
	February	May	June	July	November
Serum GH levels[1] (ng/ml)	19.1 ± 1.8^{a}	31.0 ± 2.1^{b}	68.2 ± 6.5^{c}	—	13.4 ± 1.0^{d}
Tissue irSRIF Content:[2]					
Pituitary	1010 ± 92^{a}	670 ± 85^{b}	720 ± 130^{b}	562 ± 45^{b}	1030 ± 71^{a}
Hypothalamus	$8.64\pm.74^{a}$	$6.82\pm.50^{b,c}$	$5.35\pm.36^{c}$	$6.59\pm.72^{b,c}$	$11.6\pm.87^{d}$
Telencephalon	37.0 ± 2.7^{a}	20.7 ± 2.2^{b}	$15.5\pm.79^{b,c}$	9.98 ± 1.1^{c}	46.5 ± 3.3^{d}
Thalamus+mid-brain	$2.35\pm.18^{a}$	$3.19\pm.26^{b}$	$2.37\pm.12^{a}$	$2.01\pm.17^{a}$	$4.11\pm.33^{c}$
Cerebellum+Medulla	$1.04\pm.08^{a}$	$1.55\pm.12^{b,c}$	$1.91\pm.15^{d}$	$1.21\pm.11^{a}$	$1.59\pm.12^{c,d}$

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[1] All values are represented as mean ± SEM.

[2] irSRIF in the pituitary is expressed as pg/pituitary, whereas irSRIF in the brain areas is expressed as pg/ug protein.

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4. THE EFFECTS OF GONADOTROPIN-RELEASING HORMONE ON SERUM GROWTH HORMONE LEVELS IN THE GOLDFISH

4.1 INTRODUCTION

Early studies demonstrated that extracts of the teleost hypothalamus stimulated growth hormone (GH) secretion [for review: Ball, 1981], although the nature of the active substance(s) was not known. Recently, two mammalian hypothalamic hypophysiotropic factors have been reported to elevate circulating GH levels in the goldfish. Peter _et_ _al_. [1984] reported that intraperitoneal (ip) injection of human GH-releasing hormone (GHRH) elevated serum GH levels in female goldfish, and these authors [Peter _et_ _al_., 1984] suggested that the teleost hypothalamus may contain a GH-releasing factor structurally related to the GHRHs isolated from the hypothalamus of several mammalian species [Frohman and Jansson, 1986]. However, in a preliminary report, Chang _et_ _al_. [1982] found that ip injection of an analog of mammalian gonadotropin (GTH)-releasing hormone (mGnRH) resulted in increased serum GH levels in female goldfish. This latter finding provides the possibility that GnRH may stimulate GH release in the goldfish, in addition to its well-established role as a stimulator of GTH secretion in teleost fishes [Peter _et_ _al_., 1986].

The purpose of the current study was to further examine the influences of native mammalian and teleost GnRHs, as well as analogs of these molecules, on circulating GH levels in both male and female goldfish. In addition, the influence of dopamine, a teleost GTH release-inhibitory factor [Peter et al., 1986], on GnRH-induced elevations in serum GH levels was determined by examining the influence of pimozide, a dopamine receptor antagonist, on the GnRH-induced elevation of serum GH levels.

4.2 MATERIALS AND METHODS

Experimental Animals

Goldfish of the common or comet varieties (20-30 g body weight) were purchased from Ozark Fisheries (Stoutland, MO) or Grassyforks Fisheries (Martinsville, IN). The goldfish were held in flow-through aquaria (1800 or 3600 L) at 12-15 °C under a simulated natural (Edmonton) photoperiod for at least three weeks prior to selection for the experiments. Approximately 7 days prior to an experiment, goldfish were transferred to 96 L flow-through aquaria and acclimated to a 16 h light:8 h dark (16L:8D) photoperiod and 12 °C; for Experiment 1 only, the water temperature was maintained at 18 °C. The goldfish were fed twice daily to satiation with commercially prepared fish food (Clark's New Age Fish Feed Pellets, Moore-Clark Co., LaConner, WA).

Experimental Treatments

Synthetic mammalian GnRH (mGnRH) and [D-Ala^6,Pro^{9}NEt]-mGnRH (mGnRH-A) were purchased from Sigma Chemical Co. (St. Louis, MO). Synthetic salmon GnRH ([Trp^7,Leu^8]-mGnRH; sGnRH) and [D-Arg^6,Pro^{9}NEt]-sGnRH (sGnRH-A) were generously provided by W.W. Vale and J.E. Rivier (The Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, San Diego, CA 92037). The GnRH peptides were dissolved in freshwater fish physiological saline [Burnstock, 1958] and injected ip at doses expressed as μg peptide/g body weight (injection volume = 5 μl/g body weight). Pimozide was a gift from Janssen Pharmaceuticals, Ltd. (Beerse, Belgium). Pimozide was suspended in a vehicle solution consisting of 0.7 % NaCl with 0.1 % metabisulphite and injected ip at doses expressed as μg pimozide/g body weight of fish (10 μl/g body weight).

Prior to each experiment, the fish were weighed and tagged for individual identification. Blood samples (150 to 250 μl) were taken from the caudal vasculature of anaesthetized fish using procedures described previously [Chang et al., 1985]. The blood samples were allowed to clot at 4 °C for several hours. Following centrifugation, serum from each sample was collected and stored at -28 °C. At the end of each experiment, the fish were sacrificed using excess anaesthetic, and the gonadosomatic index (GSI) of each fish was determined.

The influence of various GnRH peptides on serum GH levels was studied in Experiment 1. This experiment was conducted in late April using postovulatory females (GSI=2.3 ± 0.5) held at 18 °C. Groups of fish (n=8/group) received ip injections of either saline or 0.1 μg/g of sGnRH, sGnRH-A, mGnRH, or mGnRH-A. Blood samples were taken at 3 and 9 hours post-injection.

The effects of different dosages of mGnRH-A on serum GH levels were studied in Experiment 2. This experiment was done in late March using females with mature (preovulatory) gonads (GSI=14.8 ± 0.6) held at 12 °C. Four groups of fish (n=8/group) received an ip injection of either saline or one of three doses of mGnRH-A: 0.001, 0.01 or 0.1 μg/g. Blood samples were taken at 3, 6 and 24 hours after injection. Gonadotropin levels in serum samples from this experiment have been published elsewhere [Sokolowska et al., 1985].

The influence of various doses of pimozide on the mGnRH-A-induced elevation in serum GH levels was studied in Experiment 3. This experiment was done in late February using female goldfish in late stages of ovarian recrudescence (GSI= 10.8 ± 0.4) held at 12 °C. Groups of fish received an ip injection of either vehicle or one of three doses of pimozide (0.1, 1 or 10 μg/g) followed 3 hours later by an ip injection of saline or mGnRH-A (0.1 μg/g). Blood samples were taken at 3, 6, 24 and 48 hours after the second injection. Serum GTH levels from fish in this experiment have been published elsewhere [Sokolowska et al., 1985].

In Experiment 4, the effect of pimozide on the sGnRH-induced increase in serum GH levels in male goldfish was studied. This experiment was conducted in February using male goldfish (GSI=3.9 ± 0.2) held at 12 °C. Groups of fish (n=8/group) received an ip injection of either vehicle or pimozide (2.5 μg/g) followed 3 hours later by ip injection of saline or sGnRH (0.1 μg/g). Blood samples were taken at 3 and 24 hours after the second injection.

Growth Hormone Measurement and Satistical Analysis

Serum GH levels in Experiments 1, 2 and 3 were determined using a carp GH radioimmunoassay (RIA) previously validated for the measurement of serum GH levels in the goldfish [Cook et al., 1983]. GH levels in serum samples from Experiment 4 were analyzed using the newly developed carp GH RIA described in Appendix 1. Serum GH data were normalized using a logarithmic tranformation, and GH levels in experimental groups were compared to the GH level in the corresponding control group using Student's t-test [Snedecor and Cochran, 1980].

4.3 RESULTS

Experiment 1

At 3 hours post-injection, the groups of goldfish receiving sGnRH, mGnRH or mGnRH-A had higher serum GH levels than the saline-injected controls (Figure 4.1). At 9 hours post-injection, serum GH levels were similar in the saline- and sGnRH-injected groups; however, serum GH levels in the groups injected with mGnRH, mGnRH-A or sGnRH-A were significantly higher than in the saline control group.

The serum GH levels in the saline-injected group in Experiment 1 were higher compared to saline-injected groups in the other experiments (Figure 4.1). This is likely due to the fact Experiment 1 was conducted at 18 °C, whereas the other experiments were done at 12 °C; increased water temperature has been shown previously to result in elevated serum GH levels in the goldfish [Marchant et al., 1986].

Experiment 2

At 3 hours post-injection, each of the groups of fish receiving an injection of mGnRH-A had signficantly higher serum GH levels compared to the saline-injected group (Figure 4.2). At 6 and 24 hours after injection, serum GH levels in the groups injected with 0.001 or 0.01 µg/g mGnRH-A were not different from the levels in saline-injected controls; the serum GH levels in the group receiving 0.1 µg/g of mGnRH-A were significantly higher than the control group at both 6 and 24 hours after injection.

Experiment 3

In this experiment, female goldfish receiving an injection of vehicle followed 3 hours later by an injection of mGnRH-A (0.1 µg/g) had significantly higher serum GH levels compared to the vehicle+saline-injected control group at 3, 6 and 24 hours after the second injection (Table 4.1). Injection of 0.1, 1 or 10 µg/g of the dopamine antagonist pimozide 3 hours prior to an injection of saline did not alter serum GH levels at 3, 6 or 48 hours after the second injection, compared to the group receiving vehicle+saline (Table 4.1). At 24 hours after the second injection, the group receiving the highest dose of pimozide (10 µg/g) had a significantly elevated serum GH level compared to the control group; however, serum GH levels in the groups receiving the lower doses of pimozide prior to a saline injection had serum GH levels similar to the vehicle+saline control group.

In groups receiving an injection of pimozide followed three hours later by an injection of 0.1 µg/g mGnRH-A, serum GH levels were significantly higher at the 3 and 6 hour sample times compared to the group receiving a corresponding dose of pimozide followed by saline. At the 24 hour sample time, groups receiving the 0.1 µg/g or 1 µg/g of pimozide followed three hours later by mGnRH-A had significantly higher serum GH levels compared to the group receiving a corresponding dose of pimozide+saline. Serum GH levels at 24 hours in the group receiving 10 µg/g of pimozide+mGnRH-A were not significantly different from serum GH levels in the group injected with 10 µg/g pimozide+saline. At 48 hours after the second injection, serum GH levels in the groups receiving a 0.1 µg/g or 1 µg/g dose of pimozide followed by mGnRH-A were still significantly elevated compared to the groups receiving corresponding dosages of pimozide+saline.

With two exceptions, serum GH levels in the groups receiving the combinations of pimozide+mGnRH-A were similar to the serum GH levels in the group receiving vehicle+mGnRH-A. In the group injected with 10 μg/g pimozide followed by mGnRH-A, serum GH levels were significantly lower than in the group receiving vehicle+mGnRH-A at 6 hours after the second injection. In the group receiving 0.1 μg/g pimozide+mGnRH-A, serum GH levels were also significantly lower than the vehicle+mGnRH-A-injected group at the 24 hour sample time.

Experiment 4

Male goldfish receiving an injection of vehicle followed 3 hours later by an injection of sGnRH (0.1 μg/g) had significantly higher serum GH levels at 3 hours after the second injection compared to the control group receiving vehicle+saline injections (Figure 4.3). There were no significant differences in the serum GH levels of the pimozide+saline and the vehicle+saline groups at the 3 hour sample. The pimozide+sGnRH-injected group had serum GH levels similar to that of the group receiving vehicle+sGnRH; the serum GH levels in the pimozide+sGnRH group were significantly higher than in the pimozide+saline group. At 24 hours following the second injection, serum GH levels were similar in all four groups.

4.4 DISCUSSION

Although the stimulation of GTH secretion by GnRH is well documented in goldfish and other teleost species (see Introduction), the present report and a preliminary study by Chang *et al*., [1982] are the first to examine the influence of GnRH on circulating GH levels in a lower vertebrate. Previously, there have been several reports of mGnRH stimulating GH in release in humans with various clinical disorders: mGnRH has been reported to elevate circulating GH levels in some patients with acromegaly [Rubin *et al*., 1973; Cantalamessa *et al*., 1976], Klinefelter's syndrome [Dickerman *et al*., 1981], isolated GTH deficiency [Kasagi *et al*., 1976], schizophrenia [Gil-Ad *et al*., 1981], depression [Brambilla *et al*., 1978; Amsterdam *et al*., 1982], and heroin addiction [Brambilla *et al*., 1980]. mGnRH has also been reported to elevate plasma levels of GH in hypophysectomized rats bearing an ectopic pituitary [Panerai *et al*., 1976], and to increase GH secretion *in vitro* from rat hemipituitaries treated with enkephalin [May *et al*., 1979]. More recently, Badger *et al*. [1987] reported that mGnRH stimulated GH secretion from dispersed perifused rat pituitary cells with a potency similar to that of mammalian GRF, although the magnitude of the GH response to mGnRH was 60 % of that elicited by GRF. Thus, there is a precedent for GnRH stimulation of GH release in vertebrates, although additional studies in lower vertebrates are required before the evolutionary significance of this is known.

Previous studies in the goldfish [Peter *et al*., 1985] have shown that mGnRH-A and sGnRH-A are more active than sGnRH in elevating serum

GTH levels, whereas mGnRH and sGnRH appear to have a similar influence on serum GTH levels. In Experiment 1, serum GH levels were elevated following administration of a 0.1 μg/g dosage of all four of these GnRH peptides. However, the influence of sGnRH appears to be shorter lasting than the other three peptides; elevated serum GH levels were observed at only three hours following injection of sGnRH whereas mGnRH, mGnRH-A, and sGnRH-A stimulated increased levels for up to 9 hours. In Experiment 4, elevated circulating GH levels were observed at three hours following injection of sGnRH in male goldfish. In Experiments 2 and 3, a similar dosage of mGnRH-A caused increased circulating GH levels for up to 24 hours. These results suggest that the various analogs of GnRH (mGnRH-A and sGnRH-A), as well as mGnRH may be more active than the native teleost peptide, sGnRH, in influencing serum GH levels in the goldfish.

Results of Experiment 3 suggest that mGnRH-A elevates serum GH levels in a dose-dependent manner. At 3 hours after injection, all three doses of mGnRH-A significantly increased circulating GH levels, with the larger dosage (0.1 μg/g) being more effective that the two lower dosages (0.001 and 0.01 μg/g). Comparison of these results with serum levels of GTH measured in the same fish [GTH data published in Table 2a, Sokolowska _et_ _al_., 1985], reveals some differences. The dose-related response in GTH levels to mGnRH-A is much clearer, and the GTH response to the lower doses of mGnRH-A occurs over a longer period of time.

Several studies have provided strong evidence that dopamine functions as a GTH release-inhibitory factor in teleosts [Peter _et_ _al_., 1986]. Removal of the dopamine inhibition by injection of pharmacological agents which disrupt catecholamine synthesis or storage, or specific dopamine receptor antagonists such as pimozide have been shown to block the inhibitory actions of dopamine and to potentiate GnRH-induced GTH release [Peter et al., 1986]. For example, at 6 hours post-injection in Experiment 3, administration of the 10 μg/g dose of pimozide followed 3 hours later by injection of mGnRH-A (0.1 μg/g) resulted in a serum level of GTH approximately 6 times higher than the level measured in fish injected with mGnRH-A alone: 238±75 versus 40±8 ng GTH/ml serum, respectively [GTH data published in Table 1a, Sokolowska et al., 1985]. This potentiation by pimozide was not observed when serum GH levels were measured in the same fish; on the contrary, there was a slight but significant decrease in serum GH levels at the 6 hour sample time in the group receiving both mGnRH-A and pimozide compared to the group injected with mGnRH-A only (Table 4.1). The lack of potentiation of the actions of GnRH by pimozide on serum GH levels was also confirmed in Experiment 4 using male goldfish; at the 3 hour sampling time, serum GH levels were similar in the groups receiving sGnRH alone or in combination with pimozide.

A major difference in the neuroendocrine regulation of GH and GTH is suggested by the finding that pimozide, a dopamine antagonist, does not influence the GnRH-induced elevation of serum GH levels. Although dopamine acts as a GTH release-inhibitor in teleosts [Peter et al., 1986], a study in the goldfish [Chang et al., 1985] provided evidence suggesting that dopamine stimulates GH release, possibly by acting within the hypothalamus to inhibit somatostatin release. Previous studies have shown that somatostatin, a peptide originally isolated from the mammalian hypothalamus [Vale et al., 1977], inhibits GH release in vivo [Cook and Peter, 1984] and in vitro [Chapter 2] in the goldfish, supporting the hypothesis that somatostatin functions as a GH release-inhibitory factor in teleosts. Therefore, the release-inhibitory factors regulating GH and GTH secretion in the goldfish appear to be separate and distinct. However, the present results indicate that the regulation of GH and GTH secretion in the goldfish may occur, at least in part, through a common hypothalamic hypophysiotropic factor, GnRH.

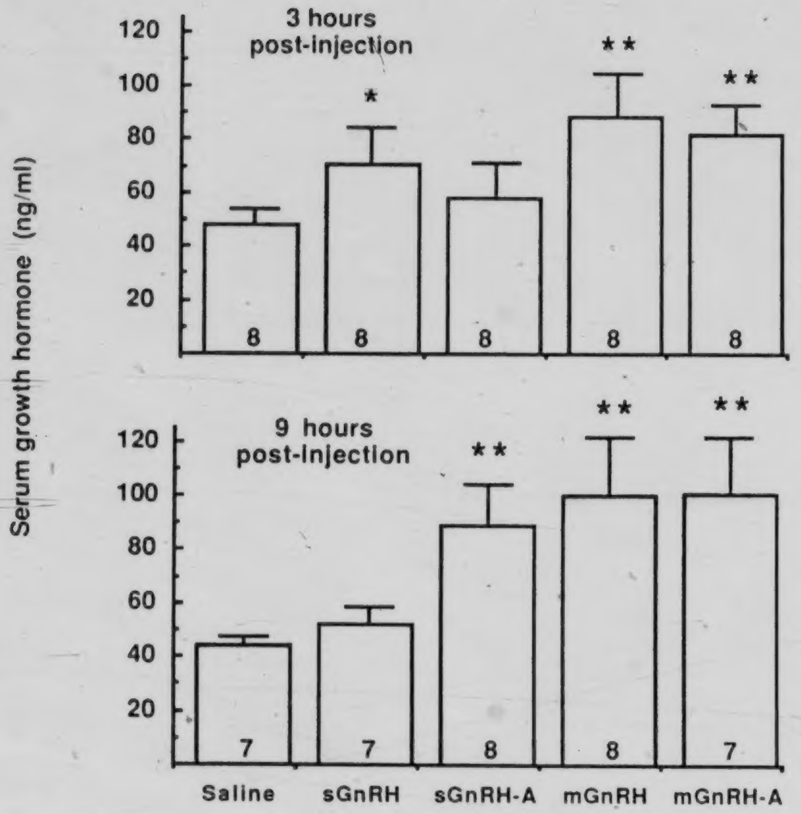

Figure 4.1. Influence of ip injection of 0.1 μg/g of sGnRH, sGnRH-A, mGnRH or mGnRH-A on serum GH levels (mean ± SEM) at 3 and 9 hours following injection in female goldfish held at 18 °C (* $p=0.07$; ** $p<0.05$ vs saline-injected group).

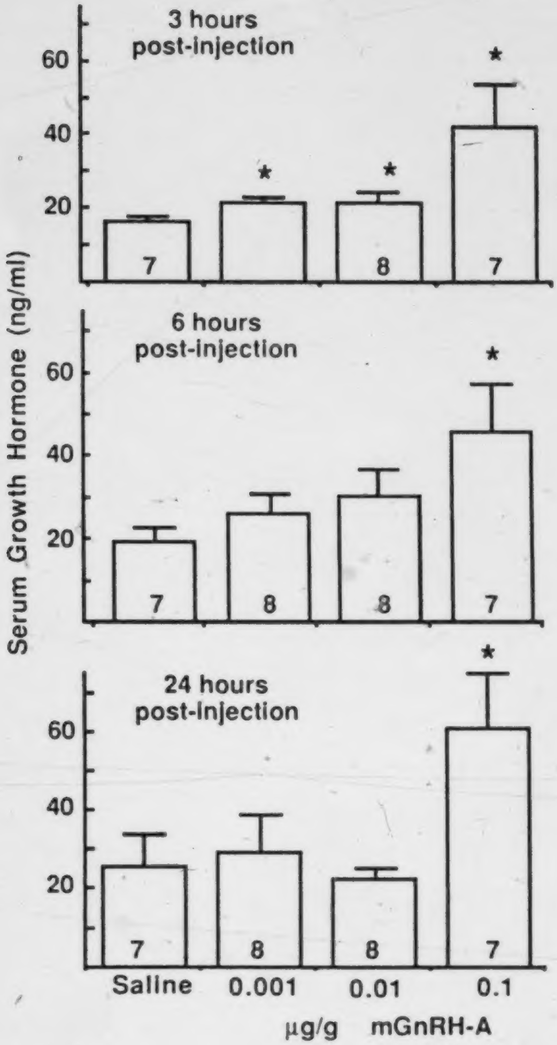

Figure 4.2. Effect of ip injection of three doses of mGnRH-A on serum GH levels (mean ± SEM) at 3, 6 and 24 hours following injection in female goldfish (* $p<0.05$ vs saline-injected group).

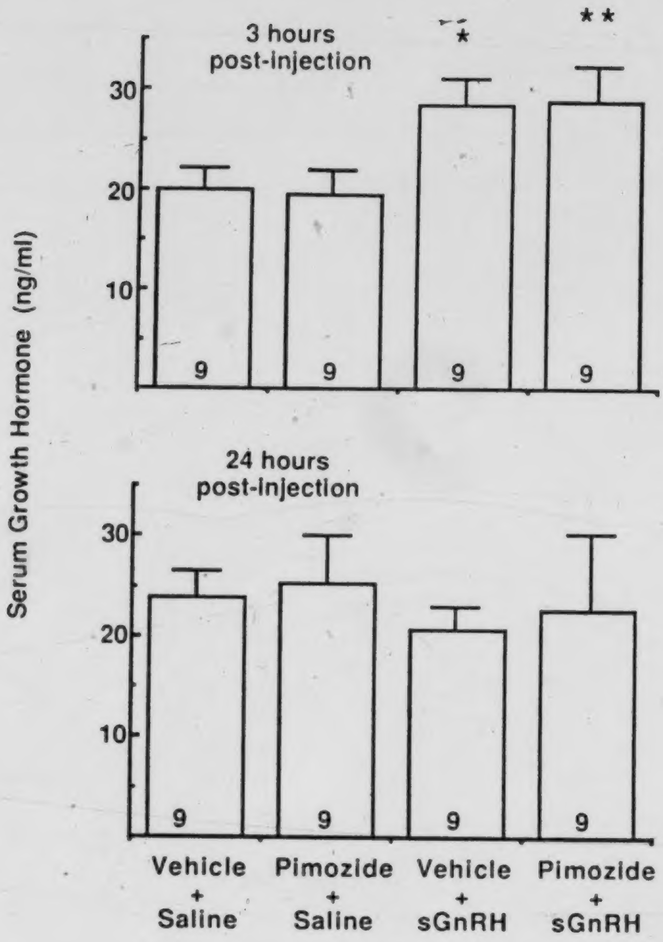

Figure 4.3. Influence of ip injection of pimozide (2.5 µg/g) followed three hours later by ip injection of sGnRH (0.1 µg/g) on serum GH levels (mean ± SEM) at 3 and 24 hours following injection in male goldfish (* $p<0.05$ vs vehicle+saline-injected group; ** $p<0.05$ vs pimozide+saline-injected group).

Table 4.1. Serum growth hormone levels in female goldfish receiving an intraperitoneal injection of vehicle or one of three dosages of pimozide, followed three hours later by an injection of saline or mGnRH-A (0.1 ug/g).

Treatment			serum Growth Hormone levels (ng/ml) at hours post-injection			
-3 hours	0 hours	n	3	6	24	48
vehicle	saline	7	19.5 ± 1.5^{1}	21.7 ± 2.0	26.5 ± 2.1	36.3 ± 6.0
pimozide (0.1 ug/g)	saline	7	19.5 ± 2.1	24.4 ± 5.1	27.0 ± 2.5	30.3 ± 4.6
pimozide (1 ug/g)	saline	7	20.3 ± 2.1	18.2 ± 2.4	23.2 ± 2.0	33.2 ± 6.1
pimozide (10 ug/g)	saline	7	23.8 ± 2.3	23.7 ± 2.4	51.4 ± 10.4^{a}	45.0 ± 4.5
vehicle	mGnRH-A	7	31.2 ± 2.8^{a}	53.7 ± 10.2^{a}	42.5 ± 2.5^{a}	65.0 ± 12.6
pimozide (0.1 ug/g)	mGnRH-A	7	38.0 ± 2.5^{b}	37.0 ± 3.0^{b}	$36.0 \pm 1.0^{b,e}$	51.9 ± 6.3^{b}
pimozide (1 ug/g)	mGnRH-A	7	49.2 ± 10.8^{c}	57.1 ± 20.0^{c}	48.2 ± 11.3^{c}	60.5 ± 12.0^{c}
pimozide (10 ug/g)	mGnRH-A	7	31.6 ± 1.3^{d}	$32.0 \pm 1.9^{d,e}$	62.4 ± 16.3	67.8 ± 12.3

[1] All values are represented as mean ± SEM.

[a] $p < .05$ compared to vehicle+saline treated group.

[b] $p < .05$ compared to pimozide (.1ug/g)+saline treated group.

[c] $p < .05$ compared to pimozide (1 ug/g)+saline treated group.

[d] $p < .05$ compared to pimozide (10 ug/g)+saline treated group.

[e] $p < .05$ compared to vehicle+mGnRH-A treated group.

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5. INFLUENCE OF GONADOTROPIN-RELEASING HORMONE ON THE SECRETION OF GROWTH HORMONE FROM FRAGMENTS OF THE GOLDFISH PITUTARY IN VITRO: COMPARISON TO GONADOTROPIN SECRETION

5.1 INTRODUCTION

Gonadotropin-releasing hormone (GnRH) has been shown to stimulate the release of gonadotropin (GTH) from the goldfish pituitary _in vitro_ [Chang _et al_., 1984; MacKenzie _et al_., 1984]. This demonstrates that GnRH acts directly at the level of the pituitary to alter GTH secretion in the goldfish. Systemic administration of various GnRH molecules were found to elevate circulating growth hormone (GH) levels in the goldfish [Chapter 4], suggesting that GnRH may also influence GH secretion in this species. However, whether this action of GnRH is due to a direct effect on GH secretion from the pituitary is not known. Therefore, in the present study, the influence of GnRH on GH secretion from the goldfish pituitary _in vitro_ was examined using perifused pituitary fragments, and compared to the influence of GnRH on GTH secretion.

5.2 MATERIALS AND METHODS

Experimental Animals

Goldfish (common or comet varieties) were purchased commercially from Grassyforks Fisheries (Martinsville, IN) or Ozark Fisheries (Stoutland, MO). The fish were maintained on a photoperiod of 16 hours light:8 hours dark in flow-through aquaria at 17 °C, and were acclimated to laboratory conditions for a minimum of two weeks prior to use in the experiments. The fish were fed to satiation twice daily with commercially prepared fish food (Clark's New Age Fish Feed Pellets, Moore-Clark Co., LaConner, WA). The goldfish used in the present study were sexually regressed (gonadosomatic index (GSI) < 0.1%), and a mixture of male and female fish were used in each experiment.

Pituitary Perifusion System

Experiments were conducted using the perifusion system previously developed for examining the release of GH [Chapter 2] and GTH [Chang et al., 1984; MacKenzie et al., 1984] from goldfish pituitary fragments. Pituitaries were collected and the pars distalis was diced into fragments (< 0.5 mm^3) using procedures described previously [Chapter 2]. Fragments of the pars distalis equivalent to 3 pituitaries were placed between two 0.1 ml layers of Cytodex carrier beads (Pharmacia, Dorval, Que.) in 0.3 ml perifusion chambers. The fragments were perifused overnight at a flow rate of 5 ml/hour with Medium 199 containing Hank's basic salts (Gibco Laboratories, Grand Island, NY) and supplemented with 25 mM HEPES buffer and Nystatin (56 U/ml). Following the overnight incubation period, the medium was changed to Hank's basic salts solution (HBSS) supplemented with 25 mM HEPES and 0.1% bovine serum albumin, with a flow rate of 15 ml/hour; the fragments were allowed to equilibrate for two hours prior to the start of the experiment. Hormone secretion from the fragments reached basal levels during the equilibration periods, and remained relatively constant in the absence of stimulation throughout the experimental period. During the experiments, five minute (1.25 ml) fractions of the medium were collected using an automatic fraction collector; fractions from six separate perifusion columns could be collected simultaneously. The fractions of perifusate were stored frozen at -25 °C prior to hormone measurement.

Experimental Protocols

The influence of prior exposure to GnRH on the subsequent hormone response to GnRH was studied in Experiments 1, 2 and 3. In Experiment 1, fragments in a total of 6 perifusion columns were exposed repeatedly to a single concentration (50 nM) of either salmon GnRH (sGnRH; n=3 columns) or the analog [D-Arg^6,Pro^{9}NEt]-sGnRH (sGnRH-A; n=3 columns). The peptide solutions were administered as three 2 minute pulses separated by 58 minute intervals. In Experiment 2, fragments in a total of 6 perifusion columns were exposed to a 2 minute pulse of 50 nM sGnRH, followed 58 minutes later by exposure to either 10 nM sGnRH (n=3 columns) or 10 nM sGnRH-A (n=3 columns) for 20 minutes; 68 minutes after the 20 minute exposure to sGnRH or sGnRH-A, the fragments in all columns were exposed to a 2 minute pulse of 50 nM sGnRH. In Experiment 3, the influence of three concentrations (1, 10 and 100 nM) of sGnRH on hormone release was studied. Fragments in individual perifusion columns were exposed sequentially to 2 minute pulses of each concentration of sGnRH administered at 58 minute intervals. In three columns, the fragments were exposed to the sGnRH solutions sequentially from the lowest to the highest concentration (ascending order), whereas in three other columns, the sGnRH solutions were administered sequentially from the highest to the lowest concentration (descending order).

In Experiment 4, the influence of sGnRH ranging in concentration from 0.01 to 1000 nM on hormone release was studied. In this experiment, fragments were exposed to 2 minute pulses of increasing concentrations of sGnRH at 58 minute intervals. Fragments in 9 columns were exposed to the following order of sGnRH doses: 0.01, 0.1, 0.5, 1, 5, 10 and 100 nM. Fragments in an additional 7 columns were exposed sequentially to the following concentrations of sGnRH: 0.25, 2.5, 25, 250 and 1000 nM.

In Experiment 5, the influence of pituitary hormone release-inhibitory factors on sGnRH-induced hormone release was studied. Fragments were exposed to two 2 minute pulses of 50 nM sGnRH at a 58 minute interval. Thirty-eight minutes after the second pulse of sGnRH, the fragments were exposed for 35 minutes to 50 nM of either somatostatin-14 (SRIF-14) or the dopamine agonist apomorphine. During exposure to SRIF-14 or apomorphine, the fragments were also exposed to a 2 minute pulse of 50 nM sGnRH. A 2 minute pulse of 50 nM sGnRH was also administered to the fragments 40 minutes after exposure to SRIF-14 or apomorphine.

Peptide Solutions

Synthetic sGnRH and sGnRH-A were generously provided by W.W. Vale and J.E. Rivier (The Clayton Foundation Laboratories for Peptide

Biology, The Salk Institute, San Diego, CA 92037). Synthetic SRIF-14 and apomorphine were purchased from Sigma Chemical Co. (St. Louis, MO). The test substances were dissolved in HBSS and were administered directly into the perifusion chambers from a separate reservoir connected to the chambers by a 3-way valve. Several concentrations of the test solutions were made immediately prior to each experiment, and kept at 4 °C until administration, at which time the temperature of the solution was allowed to equilibrate to 17 °C.

Hormone Measurements

The GH content of each fraction of perifusate was determined using a carp GH radioimmunoassay (RIA) [Appendix 1]. The GTH level in each fraction was determined using a carp GTH RIA [Peter *et al.*, 1984]. In both assays, binding inhibition curves from serial dilutions of samples of the perifusate are parallel to the standard curve, suggesting that the RIAs are suitable for measuring GH and GTH released from the goldfish pituitary *in vitro*.

Data Analysis

In Experiments 1, 2, 3 and 4, the amounts of GH and GTH in fractions collected following exposure to the GnRH solutions were expressed as a percentage of the average hormone levels in the three fractions (15 minutes) immediately preceding exposure to each GnRH solution. This transformation to % of prepulse levels allowed data from several perifusion columns to be combined for graphic presentation. In Experiments 1, 3 and 4, the hormone response to each 2 minute pulse of sGnRH or sGnRH-A was quantified by determining the average hormone level over the 25 minute period immediately following each pulse, and expressing this level as a percentage of the average hormone level over the 15 minute period immediately preceding each pulse.

In Experiment 1, differences in the rate of hormone secretion induced by the three pulses of 50 nM sGnRH or sGnRH-A were compared by analysis of variance ($p<0.05$) followed by Duncan's multiple range test ($p<0.05$) [Steel and Torrie, 1960]. In Experiment 3, differences between the rate of hormone secretion induced by each dose of sGnRH administered in either ascending or descending order were compared by analysis of variance ($p<0.05$). Following quantification of the hormone responses to the various concentrations of sGnRH in Experiment 4, data from a total of 16 columns were combined, and the dose-response curves and parameters were analyzed using the ALLFIT computer program [De Lean et al., 1978].

5.3 RESULTS

Exposure of pituitary fragments to three 2 minute pulses of 50 nM sGnRH at 58 minute intervals (Experiment 1) resulted in the rapid stimulation of both GH and GTH secretion (Figure 5.1). The rate of GH and GTH secretion over the 25 minute period immediately following exposure to sGnRH was similar for all three pulses of sGnRH (Figure 5.1). Exposure of fragments to 50 nM sGnRH-A also resulted in the rapid stimulation of the secretion of both GH and GTH (Figure 5.2). The magnitude of the hormone responses to the first pulse of sGnRH-A was significantly greater than the responses caused by the first pulse of sGnRH. Elevated hormone secretion rates following exposure to the first pulse of sGnRH-A usually lasted for a longer period of time than that caused by the first pulse of sGnRH. The secretion rates of both GH and GTH (Figure 5.2) were similar after exposure to the second and third pulses of sGnRH-A, but were significantly reduced compared to the responses caused by the first pulse of sGnRH-A.

In Experiment 2, the amount of GH and GTH in fractions collected following the administration of 2 minute pulses of 50 nM sGnRH were similar before and after exposure of the fragments to 10 nM sGnRH for 20 minutes (Figure 5.3). However, exposure of fragments to 10 nM sGnRH-A for 20 minutes reduced the secretion of GH and GTH in response to a subsequent 2 minute pulse of 50 nM sGnRH (Figure 5.3). In addition, GH and GTH secretion remained elevated following exposure of the fragments to 10 nM sGnRH-A for a longer period of time than following exposure to 10 nM sGnRH for 20 minutes.

In Experiment 3, administration of 2 minute pulses of three doses of sGnRH in ascending order of magnitude, resulted in the dose-dependent secretion of both GH and GTH (Figure 5.4). The GH and GTH secretion rates following administration of the three concentrations of sGnRH in descending order of magnitude were similar to the hormone responses elicited by the same doses administered in ascending order (Figure 5.4).

In Experiment 4, 2 minute pulses of various concentrations of sGnRH administered sequentially from the lowest to highest dose at 58 minute intervals also caused dose-dependent increases in GH and GTH secretion from pituitary fragments (Figure 5.5). In this experiment, data from several perifusion columns were combined to construct dose-response curves for the pulses of sGnRH, ranging in concentration from 0.01 to 1000 nM, for both GH and GTH secretion (Figure 5.6). Analysis of the curves using the ALLFIT computer program indicates that the half-maximal effective dose (ED_{50}) of sGnRH on GH secretion in this experiment was 2.5 ± 1.4 nM (mean ± approximate error), whereas the ED_{50} for sGnRH on GTH secretion was calculated as 6.5 ± 3.7 nM.

In Experiment 5, 2 minute pulses of 50 nM sGnRH resulted in the stimulation of both GH and GTH secretion from the fragments (Figure 5.7). However, exposure of the fragments to SRIF-14 decreased unstimulated GH secretion and completely blocked sGnRH-induced GH secretion (Figure 5.7). Subsequent exposure of the fragments to sGnRH resulted in the stimulation of GH secretion. SRIF-14 was without effect on GTH release stimulated by sGnRH. Administration of apomorphine in conjunction with a 2 minute pulse of sGnRH did not influence GH secretion induced by sGnRH. In contrast, apomorphine decreased unstimulated GTH secretion and completely blocked sGnRH-induced GTH secretion (Figure 5.7).

5.4 DISCUSSION

In a previous study, various GnRH molecules, including sGnRH and sGnRH-A, were found to elevate circulating levels of GH in both male and female goldfish [Chapter 4]. In the present study, sGnRH and sGnRH-A stimulated the release of GH from perifused fragments of the goldfish pars distalis, indicating that the GnRH molecules act directly at the level of the pituitary to alter the secretion of both GH and GTH in the goldfish. Increased GH and GTH levels were observed in the first 5 minute fraction of perifusion medium collected after exposure of the pituitary fragments to sGnRH, demonstrating that sGnRH acts rapidly to alter GH and GTH secretion. The stimulation of GH and GTH secretion by sGnRH was also dose-dependent; the ED_{50} of sGnRH on both GH and GTH secretion was in the physiological nM range.

Evidence from several experiments indicates that prior exposure of the fragments to sGnRH does not influence subsequent responses to sGnRH. For example, repeated exposure of the fragments to pulses of 50 nM sGnRH elicited an identical response after each pulse in terms of GH or GTH secretion. In another experiment, prior exposure of fragments to 10 nM sGnRH over a 20 minute period did not alter the subsequent GH and GTH responses to a 2 minute pulse of 50 nM sGnRH. Finally, the stimulation of GH and GTH secretion by three doses of sGnRH administered in ascending order of concentration was similar to the responses when the same concentrations of sGnRH were administered in descending order of concentration. Therefore, under the experimental conditions of the present study, prior exposure of the pituitary fragments to sGnRH did not alter the subsequent hormone responses to sGnRH. These findings support the validity of the experimental protocol used in the present study to determine the dose-reponse relationship between sGnRH and GH and GTH secretion from goldfish pituitary fragments.

sGnRH-A also rapidly stimulated the secretion of GH and GTH secretion from goldfish pituitary fragments. However, the profiles of the hormone responses to sGnRH-A were different than the responses to sGnRH. For example, the increases in GH and GTH secretion caused by the first pulse of sGnRH-A in Experiment 1, were greater than the increases in GH and GTH secretion measured following exposure to the first pulse of an equivalent dose of sGnRH. Furthermore, in both Experiments 1 and 2, increased secretion of GH and GTH following exposure to sGnRH-A occurred over a longer period of time than following a similar exposure to sGnRH. These findings suggest that sGnRH-A may be more active than the native molecule in stimulating hormone secretion. sGnRH-A has been shown to be more active than sGnRH _in vivo_ in elevating circulating levels of both GH [Chapter 4] and GTH [Peter _et al_., 1985] in the goldfish. Preliminary results from another _in vitro_ study using goldfish pituitary fragments also indicate that sGnRH-A is more active than sGnRH in stimulating GTH secretion [Peter _et al_., 1987], and receptor binding studies have shown that the affinity of sGnRH-A for GnRH receptors in the goldfish pituitary is greater than the affinity of sGnRH [Habibi _et al_., 1987].

In contrast to the results obtained with sGnRH, previous exposure of the fragments to sGnRH-A results in a decrease in the amount of GH and GTH released following subsequent exposure of the fragments to either sGnRH or sGnRH-A, suggesting that the pituitary fragments became less responsive to stimulation by GnRH in this situation. Studies in mammals [Catt *et al*., 1985] and birds [King *et al*., 1986] have shown that exposure of perifused pituitary cells to GnRH rapidly results in the desensitization of the gonadotrophs to further stimulation by GnRH. Interestingly, the pituitaries of two species of frogs (*Rana* sp.) were found to be relatively resistant to desensitization by GnRH [Porter and Licht, 1985], suggesting some variability among vertebrates in this phenomonon. The finding in the present study that hormone responses are reduced following previous exposure to sGnRH-A, but not sGnRH, suggests sGnRH-A desensitizes the goldfish pituitary to further stimulation. Additional studies in the goldfish are required to determine if the desensitization of the pituitary following exposure to sGnRH-A is due to alterations of the GnRH receptors or to a reduction in the amount of hormone available for release. However, comparison of the relative affinities of sGnRH and sGnRH-A for GnRH receptors on goldfish pituitary membranes [Habibi *et al*., 1987] provides the possibility that the actions of sGnRH-A observed in the present study may be related to its high affinity for pituitary GnRH receptors.

The time course of stimulation of GH secretion by sGnRH or sGnRH-A was very similar to the time course of stimulation of GTH secretion; the stimulation of GH secretion by sGnRH occurred over approximately the same time period as the stimulation of GTH secretion, and the pattern of stimulation of GH secretion in fragments previously exposed to sGnRH or sGnRH-A was very similar to that of GTH. Furthermore, the ED_{50} of sGnRH on GH and GTH release were similar: 2.5 ± 1.4 and 6.5 ± 3.7 nM, respectively. These results suggest that the receptor mechanisms for GnRH stimulation of GH and GTH secretion are similar. However, results from Experiment 5 also indicate that the actions of sGnRH on GH and GTH secretion can be separated by the administration of substances specifically inhibiting GH or GTH secretion from the goldfish pituitary. Previous studies in the goldfish provided evidence that SRIF-14 functions as a GH release-inhibitory factor [Chapters 2 and 3], whereas other studies have shown that dopamine acts as a GTH release-inhibitory factor in the goldfish [Peter et al., 1986]. In the present study, administration of SRIF-14 completely blocked the stimulation of GH secretion by sGnRH, but did not influence sGnRH-induced GTH secretion. Conversely, in the same fragments, apomorphine, a dopamine agonist, inhibited GTH secretion stimulated by sGnRH, but did not influence sGnRH-induced GH secretion. This indicates that sGnRH can stimulate the release of GH in the absence of GTH stimulation, and that GTH stimulation by sGnRH can occur in the absence of stimulated GH release.

In humans, GnRH has been shown to elevate circulating GH levels in patients with various clinical disorders [Brambilla et al., 1978, 1980; Cantelamessa et al., 1976; Dickerman et al., 1981; Gil-Ad et al., 1981; Kasagi et al., 1976]. It has been suggested that the "nonspecific" release of GH by GnRH [Brambilla et al., 1978; Panerai et al., 1976] and the "deranged anterior pituitary responsiveness" [Bramiblla et al., 1978, 1980; Dickerman et al., 1981; Panerai et al., 1976] to GnRH results from an extra-pituitary (presumably hypothalamic) action of GnRH [Brambilla et al., 1978, 1980; Dickerman et al., 1981; Gil-Ad et al., 1981; Kasagi et al., 1976; Panerai et al., 1976]. It has also been suggested that the influence of GnRH on GH release may be due to the presence of "abnormal" receptors on the somatotrophs [Cantelamessa et al., 1976; May et al., 1979; Kasagi et al., 1976]. Recently, it was reported in an abstract that mammalian GnRH stimulates GH secretion in dispersed perifused rat pituitary cells with a potency similar to that of mammalian GH-releasing hormone [Badger et al., 1987], indicating that GnRH may also stimulate GH secretion from normal mammalian pituitary cells. The results of the present study strongly indicate that GnRH acts directly at the level of the pituitary in the goldfish, and that the effects of GnRH on GH and GTH release may be mediated through a similar receptor response. Therefore, the results of this study may provide an evolutionary basis for the "anomolous" stimulation of GH secretion by GnRH in mammals. Findings of the present study provide evidence suggesting that the release-inhibitory factors regulating GH and GTH secretion in the goldfish are separate and distinct, but that the secretion of both GH and GTH may be regulated at least in part through a common hypothalamo-hypophyseal releasing factor, GnRH.

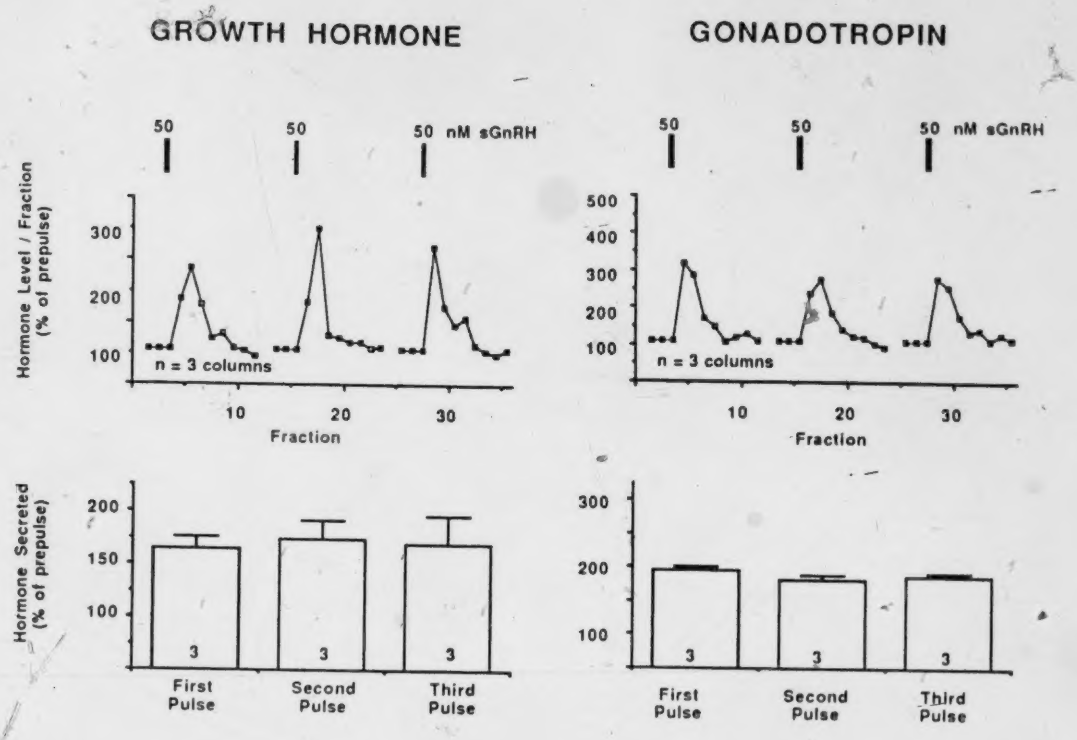

Figure 5.1. Influence of exposure to three two minute pulses (black bars) of 50 nM sGnRH on the secretion of GH (left panels) and GTH (right panels) from perifused goldfish pituitary fragments. The average hormone levels in fractions from three separate perifusion columns are presented, and are expressed as a % of the average hormone levels in the three fractions immediately preceding each pulse of sGnRH (% of prepulse). In these three columns, the average secretion rate of GH and GTH over the initial 15 minute period prior to exposure of the fragments to the peptide solutions was 42.3 and 13.7 ng/fraction, respectively. The amounts of GH and GTH secreted over the 25 minute period following each pulse are shown in the bottom panels (mean ± SEM, n=3 columns), and are expressed as a % of the average amounts of hormone secreted during the 15 minute period preceding each pulse (% of prepulse).

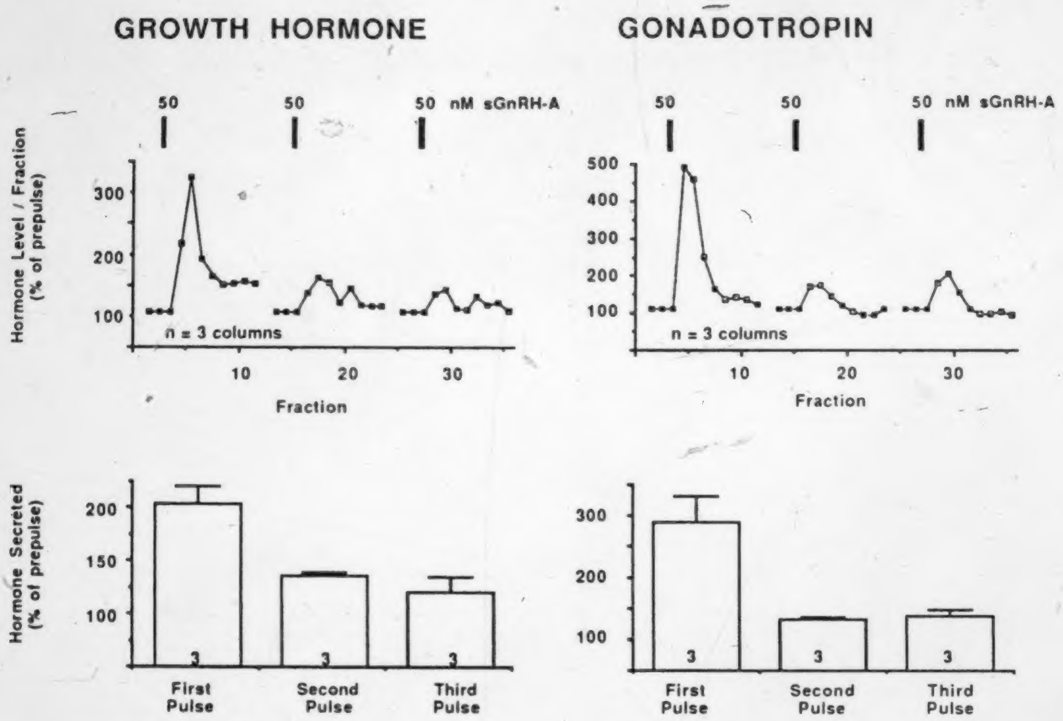

Figure 5.2. Influence of exposure to three two minute pulses (black bars) of 50 nM sGnRH-A on the secretion of GH (left panels) and and GTH (right panels) from perifused goldfish pituitary fragments. See Figure 5.1 legend for further details regarding the presentation of the data. In these three columns, the average secretion rate of GH and GTH over the initial 15 minute period prior to exposure of the fragments to the peptide solutions was 30.6 and 13.1 ng/fraction, respectively.

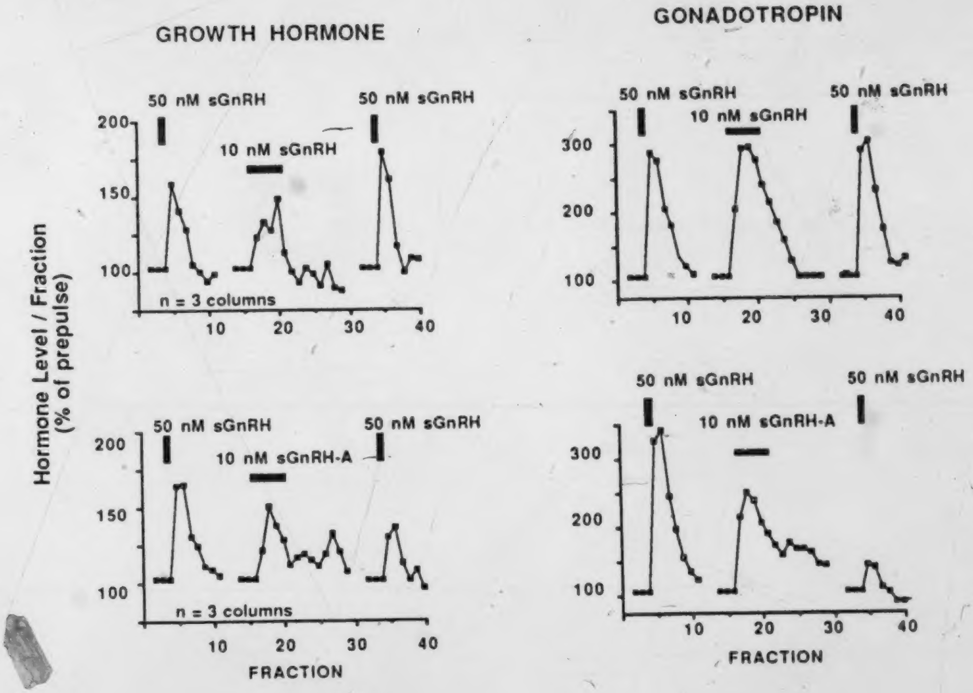

Figure 5.3. Influence of exposure to 10 nM sGnRH (top panels) or sGnRH-A (bottom panels) for 20 minutes (horizontal bars) on the subsequent stimulation by a two minute pulse of 50 nM sGnRH (vertical bars) of GH (left panels) and GTH (right panels) from perifused goldfish pituitary fragments. In each panel, the average hormone levels from three separate perifusion columns are presented, and are expressed as % of the average hormone levels in the 3 fractions immediately preceding exposure to each of the peptide solutions. In the six columns, the average secretion rate of GH and GTH over the initial 15 minute period prior to exposure of the fragments to the peptide solutions was 41.2 and 55.7 ng/fraction, respectively.

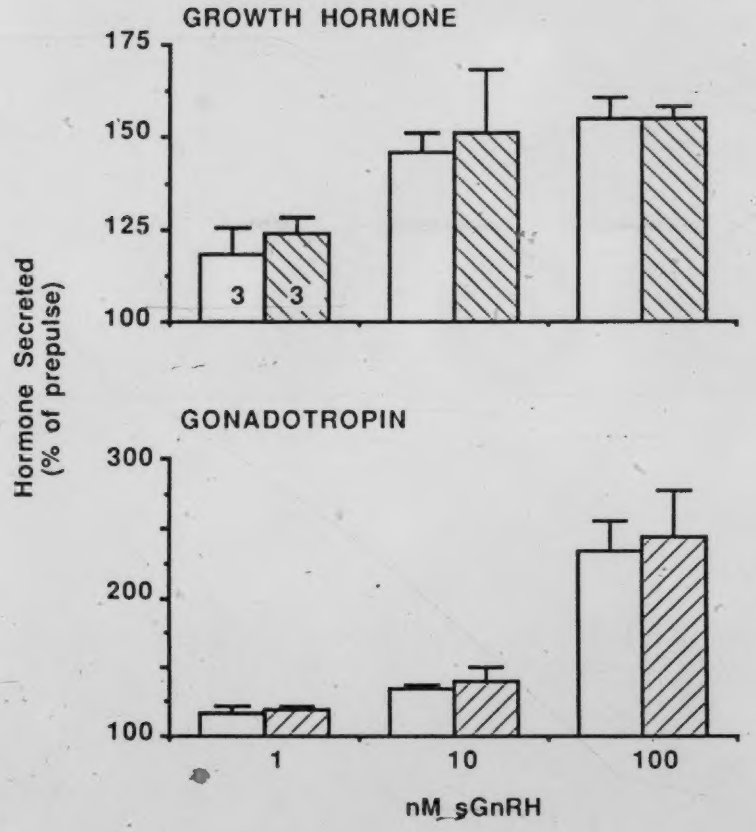

Figure 5.4. Influence of exposure to two minute pulses of three concentrations of sGnRH on the secretion of GH and GTH from perifused goldfish pituitary fragments. The three doses of sGnRH were administered at 60 minute intervals in either ascending order of increasing concentration (open bars) or descending order of concentration (hatched bars). The amounts of GH and GTH secreted during the 25 minute period following each dose of sGnRH are expressed as a % of the amount of each hormone secreted over the 15 minute period preceding each pulse (% of prepulse). The values represent mean ± SEM from three separate perifusion columns. In these columns, the average secretion rate of GH and GTH over the initial 15 minute period prior to exposure of the fragments to the peptide solutions was 44.5 and 15.8 ng/fraction, respectively.

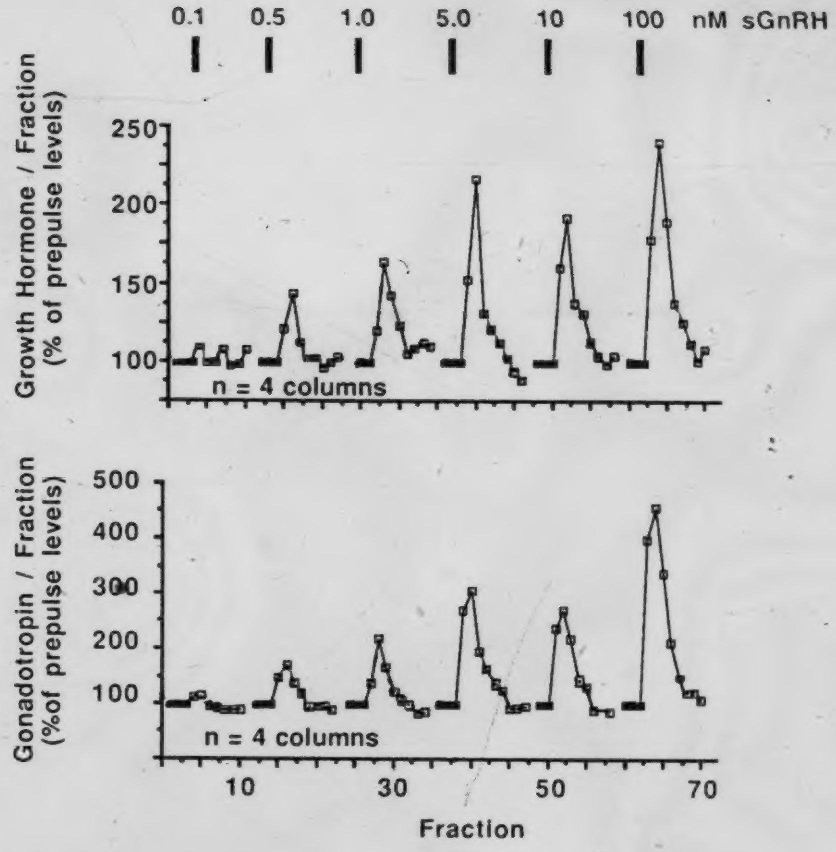

Figure 5.5. Representative profiles of GH (top panel) and GTH (bottom panel) levels in fractions collected following exposure of goldfish pituitary fragments to two minute pulses (black bars) of various concentrations of sGnRH at 60 minute intervals. Average hormone levels in fractions from 4 separate perifusion columns are presented, and are expressed as a % of average hormone levels in the three fractions immediately preceding each pulse (% of prepulse). In these 4 columns, the average secretion rate of GH and GTH over the initial 15 minute period prior to exposure of the fragments to the peptide solutions was 31.6 and 32.1 ng/fraction, respectively.

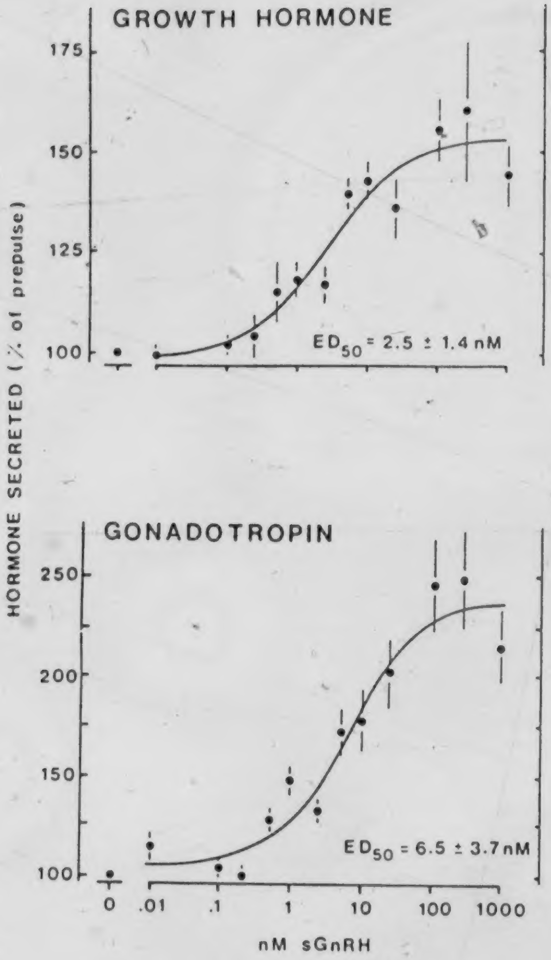

Figure 5.6. Dose response curves for sGnRH on the stimulation of GH (top panel) and GTH (bottom panel) secretion from perifused goldfish pituitary fragments. The curves were determined by exposing perifused pituitary fragments to 2 minute pulses of various concentrations of sGnRH, and were analyzed using the ALLFIT computer program (see Materials and Methods for a detailed decription). Values are represented as mean ± SEM (n=7 to 9). The ED$_{50}$ (mean ± approximate error) of sGnRH on GH and GTH secretion are indicated in each panel.

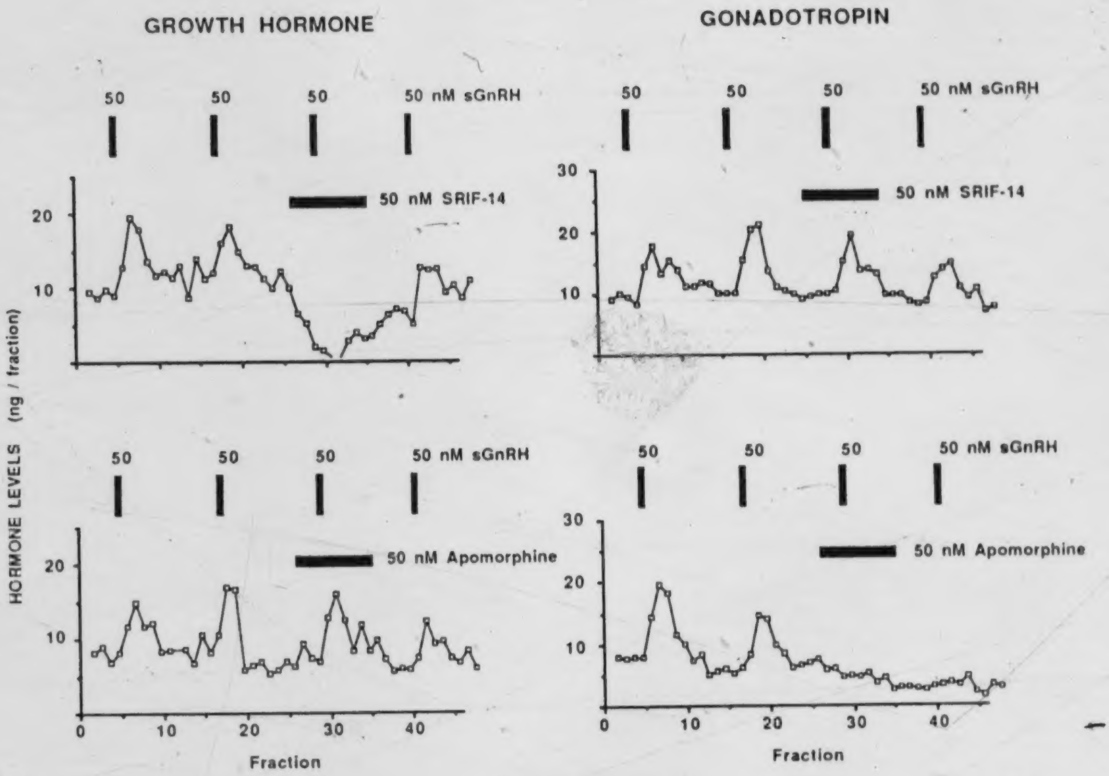

Figure 5.7. Influence of 50 nM SRIF-14 or apomorphine (horizontal bars) on the levels of GH (left panels) and GTH (right panels) in perifusion medium collected following exposure of goldfish pituitary fragments to two minute pulses of 50 nM sGnRH (vertical bars).

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6. THE INFLUENCE OF AN ANALOG OF MAMMALIAN GONADOTROPIN-RELEASING HORMONE ON THE RATE OF BODY GROWTH IN GOLDFISH

6.1 INTRODUCTION

In vivo [Chapter 4] and *in vitro* [Chapter 5] experiments suggest that gonadotropin (GTH)-releasing hormone (GnRH) functions as a growth hormone (GH)-releasing factor in the goldfish. For example, intraperitoneal (ip) injection of the analog [D-Ala^6,Pro^{9}NEt]-mammalian GNRH (mGnRH-A) elevates serum GH levels for up to 24 hours following injection in female goldfish (Table 5.1). Previous studies in the goldfish have related increased serum GH levels to increased rates of body growth [Cook and Peter, 1983; Marchant and Peter, 1986; Marchant *et al*., 1986], and it is hypothesized that the GnRH-induced increase in serum GH levels is also sufficient to increase the rate of body growth in the goldfish. This possibility was examined in the present study by measuring the rate of body growth in goldfish receiving repeated ip injections of mGnRH-A.

6.2 MATERIALS AND METHODS

Experimental Animals

Goldfish of the common or comet varieties were purchased from Ozark Fisheries (Stoutland, MO). On arrival, the goldfish were placed in 96 L flow-through aquaria and maintained on a photoperiod of 16 hours light:8 hours dark (16L:8D) at a water temperature of 14 ± 1 °C. The goldfish were allowed to acclimate to laboratory conditions for a minimum of four weeks prior to the experiments. For each experiment, fish were fin-clipped for individual identification, and fish in the treatment groups were divided between two 96 L aquaria. The fish were allowed to acclimate for an additional five day period at 16L:8D and 14 °C. During the acclimation and experimental periods, the fish were fed to satiation twice daily with commercially prepared fish food (Clark's New Age Fish Feed Pellets, Moore-Clark Co., LaConner WA). Food was withheld on the mornings of treatment in the experiment. Prior to handling, the fish were lightly anaesthetized in 0.05% tricaine methanesulphonate.

Experimental Protocols

Synthetic mGnRH-A was purchased commercially (Syndel Laboratories, Vancouver, B.C.), and dissolved in saline (0.6 % NaCl) supplemented with Penicillin G (260 U/ml). mGnRH-A was injected ip at doses expressed as μg mGnRH-A/g body weight (injection volume = 5 μl/g). Control animals were injected with an equivalent volume of saline.

In experiment 1, the influence of two doses of mGnRH-A on the rate of body growth in female goldfish was examined. Three groups of fish received a total of 5 injections of either saline, 0.01 µg/g mGnRH-A, or 0.1 µg/g mGnRH-A at three day intervals. On the day of the first injection (Day 0), the weight of each fish was measured to the nearest decigram following gentle blotting on damp paper towelling to remove excess surface water. The body length of each fish on Day 0 was measured from the tip of the snout to the end of the caudal peduncle using calipers accurate to the nearest decimillimeter. The average weight and length on Day 0 of the fish used in Experiment 1 was 5.52 ± 0.09 g and 5.21 ± 0.03 cm (mean ± SEM; n=27), respectively. At the end of the experiment (Day 16), the final body weight and length of each fish was measured, the fish sacrificed by spinal transection, and the weight of the gonad from each fish determined.

In experiment 2, three groups of female goldfish received a total of 6 injections of either saline, 0.01 µg/g mGnRH-A or 0.1 µg/g mGnRH-A at 5 day intervals. The body weight and length was determined at the start (Day 0) and the end (Day 30) of the experiment using procedures similar to those described for Experiment 1. The average body weight and length of the fish on Day 0 was 7.78 ± 0.18 g and 5.67 ± 0.05 cm (mean ± SEM, n=30), respectively. Gonad weights were measured following sacrifice on Day 30.

In Experiment 3, the influence of rabbit antisera generated against carp GTH [Peter _et_ _al_., 1984] and carp GH [Appendix 1] on mGnRH-A-induced growth was examined using male goldfish. Six groups of goldfish received a total of 4 injections at five day intervals of either saline or mGnRH-A (0.1 µg/g) concurrently with ip injections (25 μl/fish) of either normal rabbit serum (NRS), rabbit anti-carp GTH serum (racGTH), or rabbit anti-carp GH serum (racGH). The body weight and length of each fish at the start (Day 0) and end (Day 20) of Experiment 3 was measured as described for Experiment 1. The average body weight and length of the fish on Day 0 was 7.73 ± 0.2 g and 5.88 ± 0.05 cm (mean ± SEM, n=48), respectively. The weight of the gonad from each fish was measured following sacrifice on Day 20.

Data Analysis

The growth rate of individual fish in each experiment was calculated as the % increase in body weight and length over the experimental period. Growth rate data were normalized using a logarithmic transformation, and differences in growth rates between groups were analyzed using Student's t-test [Snedecor and Cochran, 1980]. Gonadal weights were calculated as % of body weight (gonadosomatic index, GSI).

6.3 RESULTS

In Experiment 1, the increase in body length over the 16 day period was significantly greater in fish receiving 0.1 µg/g mGnRH-A compared to the control group receiving saline (Figure 6.1). All groups displayed similar increases in body weight over the experimental period (Figure 6.1); the GSI was also similar in all three groups (Figure 6.1).

In Experiment 2, the groups of fish receiving either dose of mGnRH-A (0.01 or 0.1 µg/g) had significantly greater increases in body length over the 30 day experimental period than the group receiving saline (Figure 6.2). The increases in body weight over the experimental period and the GSI values were similar in all three groups in Experiment 2 (Figure 6.2).

In Experiment 3, the groups of male fish receiving mGnRH-A (0.1 µg/g) in combination with either NRS or racGH displayed significantly greater increases in body length over the 20 day experimental period compared to the corresponding control groups receiving saline in combination with either NRS or racGH (Table 6.1); the increase in body length in the group receiving mGnRH-A plus racGTH also tended to be higher than in the group receiving saline plus racGTH ($p=0.08$; Table 6.1). The increase in body length was similar in all three groups receiving saline during the experimental period. The group receiving mGnRH-A plus racGTH had an increase in body length similar to that of the group injected with mGnRH-A plus NRS, whereas the group receiving mGnRH-A plus racGH displayed an increase in body length that tended ($p=0.10$) to be lower than that of fish receiving mGnRH-A plus NRS (Table

6.1). The % increase in body weight over the experimental period and the GSI values were similar in all groups in Experiment 3 (Table 6.1).

6.4 DISCUSSION

In the present study, repeated ip injection of mGnRH-A at 3 or 5 day intervals stimulated body growth in both female and male goldfish, as indicated by increases in body length following treatment with mGnRH-A. In Experiment 1, only the highest dose of mGnRH-A (0.1 μg/g) was effective in increasing body length over the 16 day treatment period, although a lower dose of mGnRH-A (0.01 μg/g) also increased body length over a 30 day treatment period (Experiment 2). In all experiments, the increase in body length following mGnRH-A treatment was independent of changes in total body or gonadal weights.

In Experiment 3, injection of an antiserum against carp GH concurrently with the injections of mGnRH-A partially blocked the growth response to mGnRH-A in male goldfish; injection of this antiserum at a dose of 50 μl/fish has been shown previously to suppress normal growth in female goldfish [Appendix 1]. Notably, in the same experiment, concurrent injection of antiserum against carp GTH did not influence the mGnRH-A-induced increase in body length. These results indicate that body growth induced by mGnRH-A in the goldfish is at least partially dependent on circulating GH levels. Previously, a single ip injection of mGnRH-A (0.1 μg/g) was found to stimulate a two-fold increase in serum GH levels for up to 24 hours following injection [Chapter 4]. The present study provides evidence that increases in serum GH levels induced by mGnRH-A are sufficient to stimulate growth in the goldfish.

The present study is the first to demonstrate increased body growth in a teleost species following administration of a hypothalamic peptide stimulating endogenous GH secretion. Although there has been considerable interest in the use and potential aquacultural applications of exogenously administered GH as a growth promoter in a variety of teleost species [Donaldson et al., 1979], only a few studies [Cook and Peter, 1983; Pickford et al., 1981] have measured body growth in teleosts following alterations to neuorendocrine mechanisms regulating endogenous GH levels. However, in other vertebrate species such as humans [Frohman and Jansson, 1986; Gelato and Merriam, 1986], rats [Clark and Robinson, 1985; Wehrenberg, 1986], transgenic mice [Hammer et al., 1985] and chickens [Leung et al., 1986], increased body growth has been achieved with the use of mammalian GH-releasing factor, a hypothalamic peptide stimulating GH secretion in mammals [Frohman and Jannson, 1986] and birds [Scanes et al., 1986]. Results of the present study in goldfish indicate that manipulation of the neuroendocrine mechanism(s) regulating endogenous GH secretion may also be used successfully to enhance body growth in teleost fishes.

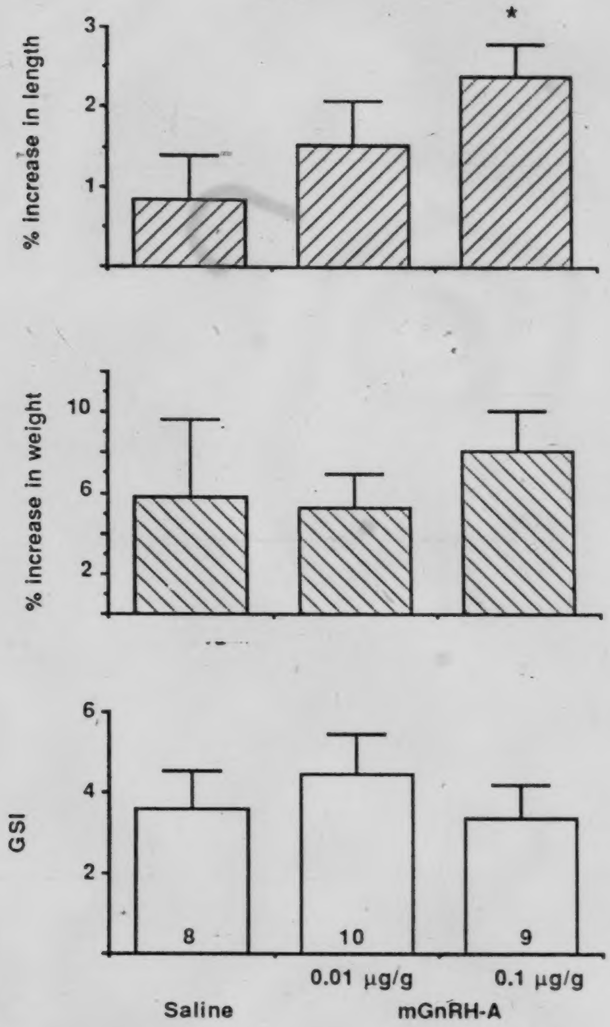

Figure 6.1. Increases in body length (top panel) and body weight (middle panel) in groups of female goldfish receiving four injections, at three day intervals, of either saline, 0.01 µg/g mGnRH-A or 0.1 µg/g mGnRH-A. The gonadosomatic index (GSI) of the three groups measured at the end of the treatment period (Day 16) are also indicated (bottom panel). All values are mean ± SEM; sample sizes are indicated in the lower panel. Significant ($p<0.05$) differences compared to the saline treated group are indicated by an asterisk.

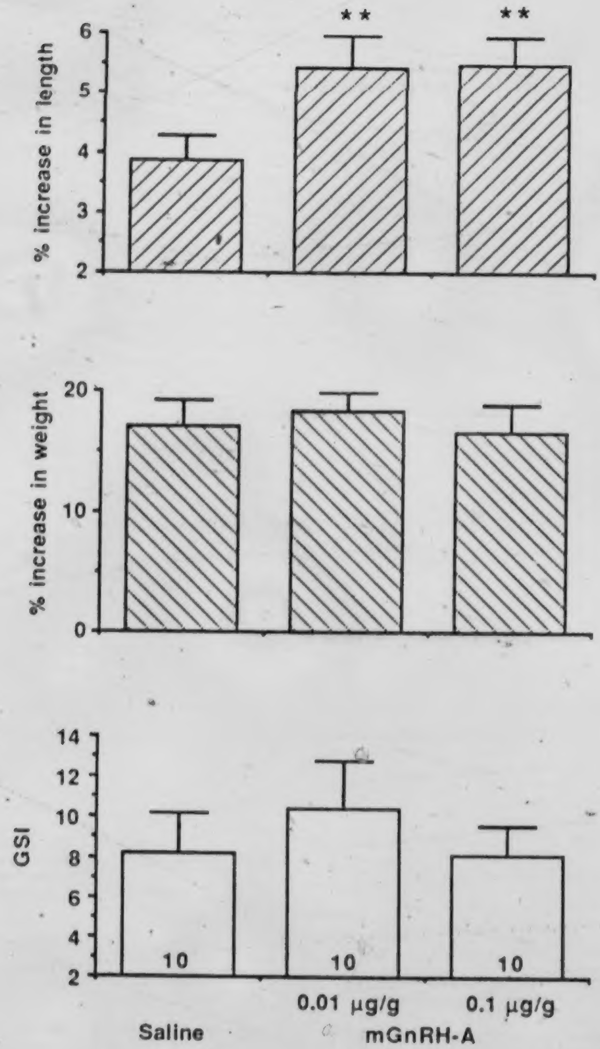

Figure 6.2. Increases in body length (top panel) and body weight (middle panel) in groups of female goldfish receiving six injections, at five day intervals, of either saline, 0.01 µg/g mGnRH-A or 0.1 µg/g mGnRH-A. The gonadosomatic index (GSI) of the three groups meaured at the end of the treatment period (Day 30) are also indicated in the lower panel. All values are mean ± SEM; sample sizes are indicated in the lower panel. Significant ($p<0.05$) differences compared to the saline treated group are indicated by an asterisk.

Table 6.1. Influence of four ip injections, at 5 day intervals, of normal rabbit serum (NRS), rabbit anti-carp GTH serum (racGTH) or rabbit anti-carp GH serum (racGH) injected concurrently with saline or mGnRH-A (0.1 μg/g) on body growth in male goldfish over the 20 day treatment period. Growth rates are expressed as the % increase in body length or weight over the treatment period. Gonad weights measured at the end of the experiment (Day 20) are expressed as % of body weight (gonadosomatic index; GSI).

TREATMENT	n	LENGTH (% increase)	WEIGHT (% increase)	GSI
Saline + NRS	8	-0.27±0.58[1]	2.02±2.6	2.10±0.53
Saline + racGTH	8	0.20±0.51	6.78±2.5	3.34±0.33
Saline + racGH	8	-0.63±0.35	2.04±1.2	2.14±0.47
mGnRH-A + NRS	8	1.68±0.32[2]	6.33±1.7	2.86±0.84
mGnRH-A + racGTH	8	1.64±0.58[3]	5.73±2.5	3.87±0.35
mGnRH-A + racGH	8	0.88±0.34[4] [5]	6.58±2.7	2.77±0.59

[1] All values are mean ± SEM.
[2] $p<0.01$ versus Saline + NRS.
[3] $p=0.08$ versus Saline + racGTH.
[4] $p<0.01$ versus Saline + racGH.
[5] $p=0.10$ versus mGnRH-A + NRS.

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7. INFLUENCE OF HUMAN GROWTH HORMONE-RELEASING HORMONE (GHRH) AND A CARP GHRH-LIKE IMMUNOREACTIVE PEPTIDE ON GROWTH HORMONE SECRETION IN THE GOLDFISH

7.1 INTRODUCTION

A peptide stimulating growth hormone (GH) secretion from the mammalian pituitary has been isolated from the hypothalamus of several mammalian species [for review: Frohman and Jansson, 1986; Gelato and Merriam, 1986]. In most species, the hypothalamic GH-releasing hormone (GHRH) is an amidated 44 amino acid peptide, although in the rat, the GHRH is a non-amidated 43 amino acid peptide. Mammalian GHRH also displays other structural variations between species. For example, rat GHRH displays only 70 % homology with human GHRH. Human GHRH also appears to exist in two forms: as a 44 residue peptide (hGHRH$_{1-44}$), and as a smaller molecule (hGHRH$_{1-40}$) with a peptide sequence corresponding to the first 40 residues of hGHRH$_{1-44}$. Recently, a 45 amino acid peptide resembling mammalian GHRH in structure has been isolated from hypothalamic extracts of a teleost species, the common carp *Cyprinus carpio* [Vaughan *et al*., 1987; J. Rivier and W. Vale, personal communication]. The carp GHRH-like immunoreactive peptide (carp GHRH-LIP$_{1-45}$) was isolated based on its immunological cross reactivity with an antiserum against rat GHRH, and sequence analysis has shown that the carp GHRH-LIP has partial sequence homology to mammalian GHRH [Vaughan *et al*., 1987]. Two subforms of carp GHRH-LIP$_{1-45}$ have also been isolated from the carp hypothalamic extracts: carp GHRH-LIP$_{1-2}$, and carp

GHRH-LIP$_{1-44}$) with peptide sequences corresponding to the first 29 and 44 amino acids, respectively, of carp GHRH-LIP$_{1-45}$ (J. Rivier and W. Vale, personal communication).

A large number of studies in a variety of mammalian species, have demonstrated that mammalian GHRH rapidly stimulates GH secretion *in vivo* and *in vitro* in a potent and dose-dependent manner [for review: Frohman and Jansson, 1986; Gelato and Merriam, 1986]. Mammalian GHRH has also been shown to stimulate GH release *in vivo* and *in vitro* in a variety of avian species [for review: Scanes *et al*., 1986], and it is hypothesized that a molecule very similar in structure to mammalian GHRH participates in the regulation of GH secretion in birds. In lower vertebrates, the hypothalamic regulation of GH secretion has not been extensively studied, and the possible role of a GHRH-like molecule in the hypothalamic regulation of GH release in poikilothermic vertebrates is virtually unstudied. A single report in the goldfish [Peter *et al*., 1984] demonstrated that intraperitoneal (ip) injection of hGHRH$_{1-40}$ resulted in a significant elevation in serum GH levels, and these authors suggested that GH secretion in the goldfish may be regulated at least partially through a hypothalamic peptide structurally related to the human GHRH molecule. Immunoreactive perikarya and nerve fibers have also been demonstrated in the brain of the codfish (*Gadus morhua*) in immunocytochemical studies using antisera against the human GHRHs [Pan *et al*., 1985a; Pan *et al*., 1985b]. However, the influence of mammalian GHRH on GH secretion *in vitro* from the pituitary of the goldfish or other teleost species has not been reported, and a direct action of the mammalian GHRH on the teleost pituitary to stimulate GH secretion remains to be demonstrated. Furthermore, the influence of the GHRH-like molecule recently isolated from the carp on GH secretion in a teleost species remains unknown.

The present study examined the influence of hGHRH$_{1-40}$ and hGHRH$_{1-44}$ on circulating GH levels in the goldfish, and on the secretion of GH _in vitro_ from fragments of the goldfish pituitary gland maintained in a perifusion system. The effect of the various forms of carp GHRH-LIP on GH secretion _in vitro_ was also studied, and compared to the influence of salmon gonadotropin-releasing hormone (sGnRH) [Chapter 5] on GH secretion.

7.2 MATERIALS AND METHODS

Experimental Animals

Goldfish (common or comet varieties) were purchased commercially (Grassyforks Fisheries, Matinsville, IN or Ozark Fisheries, Stoutland, MO), and placed in 96 L flow-through aquaria. For the *in vivo* experiments, female goldfish were allowed to acclimate to laboratory conditions for a minimum of four weeks prior to the start of the experiments. Five days before each experiment, the goldfish (20 to 30 g) were tagged for individual identification and acclimated to a photoperiod of 16 hours light:8 hours dark (16L:8D) at a water temperature of either 12 °C (Experiments 1, 2 and 3) or 20 °C (Experiment 1). For the *in vitro* experiments, goldfish of mixed sex were acclimated for a minimum of two weeks to a photoperiod of 16L:8D and a water temperature of 17 °C. Throughout the acclimation periods, the fish were fed to satiation twice daily with commercially prepared fish food (Clark's New Age Fish Feed Pellets, Moore-Clark Co., LaConner WA); during the *in vivo* experiments, food was withheld on the day of injection and blood sampling. Prior to injection, blood sampling or sacrifice in all experiments, the fish were anaesthetized in 0.05 % tricaine methanesulphonate.

In Vivo Experiments

In Experiment 1, the influence of hGHRH_{1-40} and somatostatin-14 (SRIF-14) on circulating GH levels in female goldfish acclimated to either 12 or 20 °C was studied. This experiment was conducted in

November using fish in the early stages of gonadal recrudescence. All peptides were dissolved in saline (0.6% NaCl) and injected ip at dosages expressed as µg peptide/g body weight (injection volume = 5 µl/g). At each temperature, two groups of fish received a single ip injection of $hGHRH_{1-40}$ at a dosage of either 0.1 or 1.0 µg/g. A third group of fish at each temperature received a single injection of SRIF-14 (1.0 µg/g). Control fish received an equivalent volume of saline. Blood samples were obtained from the caudal vasculature using procedures described previously [Chang _et_ _al_., 1985]. The blood samples were allowed to clot at 4 °C for several hours, and the serum from each sample was collected and stored at -25 °C. All fish were sacrificed following the final blood sample.

Experiment 2 was conducted in May using female goldfish held at 12 °C with mature (preovulatory) gonads. Groups of fish received a single injection of either saline, or one of three concentrations of $hGHRH_{1-40}$: 0.01, 0.1 or 1.0 µg/g. Blood samples were obtained at 1, 3 and 6 hours after the final blood sample.

Experiment 3 was conducted in April using female goldfish in the final stages of gonadal recrudescence held at 12 °C. Groups of fish received a single injection of either saline, $hGHRH_{1-44}$ (1.0 µg/g) or sGnRH (0.1 µg/g). Blood samples were taken at 1, 3 and 8 hours after the final blood sample.

_In_ _Vitro_ _Experiments_

The influence of various GHRH-like peptides on GH secretion from fragments of the goldfish pars distalis was examined using pituitary perifusion techniques previously used to study the influence of various

SRIF [Chapter 2] and GnRH [Chapter 5] molecules on GH release from the goldfish pituitary. In perifusion Experiment A, fragments of the pars distalis equivalent to 5 pituitaries were placed in perifusion chambers and allowed to equilibrate for a period of two hours prior to the start of the experiment. The perifusion medium during the equilibration and experimental periods consisted of Hank's basic salt solution (HBSS) supplemented with 25 mM HEPES buffer and 0.1 % bovine serum albumin [Chapter 2]. The flow rate of HBSS through the perifusion column was maintained at 15 ml/hour. After the two hour equilibration period, fractions of the perifusate were collected at 5 minute (1.25 ml) intervals using an automatic fraction collector. At the start of the experimental period, fractions were collected over a 40 minute period, prior to administration of the peptide solutions. The fragments were then exposed to three concentrations (1, 10 and 100 nM) of $hGHRH_{1-40}$. The three dosages of $hGHRH_{1-40}$ were adminstered sequentially from the lowest to the highest concentration, and each dosage was adminstered over a 20 minute period. Following exposure to 100 nM $hGHRH_{1-40}$, the fragments were exposed to HBSS only for a 60 minute period. Three concentrations (1, 10 and 100 nM) $hGHRH_{1-44}$ were then adminstered sequentially from the lowest to the highest dosage; each dosage of $hGHRH_{1-44}$ was administered over a 20 minute period. Finally, the fragments were exposed to HBSS only for a 40 minute period following administration of the 100 nM dosage of $hGHRH_{1-44}$. This experimental protocol was tested in four separate perifusion columns.

In perifusion Experiment B, fragments equivalent to 3 pars distalis were placed in perifusion chambers and allowed to equilibrate overnight prior to the start of the experimental treatments. During the overnight equilibration period, the flow rate of medium through the columns was 5 ml/hour and the perifusion medium consisted of Medium 199 containing Hank's basic salts (Gibco Laboratories, Grand Island, NY) and supplemented with 25 mM HEPES buffer and Nystatin (56 U/ml). Two hours prior to the start of the experimental period, the flow rate was increased to 15 ml/hour and the medium was changed to HBSS. Five minute (1.25 ml) fractions were collected during the experimental period. In this experiment, pituitary fragments were exposed to discrete 5 minute pulses of various concentrations of either hGHRH$_{1-44}$, an analog of human GHRH ([Nle^{27}]-human GHRH(1-29); hGHRH-A), carp GHRH-LIP$_{1-45}$, carp GHRH-LIP$_{1-44}$, or carp GHRH-LIP$_{1-29}$. For hGHRH$_{1-44}$, hGHRH-A, carp GHRH-LIP$_{1-44}$ and carp GHRH-LIP$_{1-29}$, three concentrations (10, 100 or 1000 nM) of each peptide were administered sequentially from the lowest to the highest dose as discrete 5 minute pulses at 30 minute intervals; each peptide was tested in individual perifusion columns. Forty-five minutes after the pulse of the 1000 nM concentration of each of these peptides, the fragments in all perifusion columns were exposed to a 2 minute pulse of 50 nM sGnRH. For carp GHRH-LIP$_{1-45}$, 50 and 500 nM concentrations of the peptide were adminstered as discrete 5 minute pulses at a 55 minute interval. A 2 minute pulse of 50 nM sGnRH was administered 58 minutes after exposure to the 500 nM dosage of carp GHRH-LIP$_{1-45}$. Each peptide was tested on pituitary fragments in at least two individual perifusion columns. All fractions of perifusate were stored frozen at -25 °C until assayed for GH content.

Peptide Solutions

Synthetic $hGHRH_{1-40}$, $hGHRH_{1-44}$, hGHRH-A, carp $GHRH\text{-}LIP_{1-45}$, carp $GHRH\text{-}LIP_{1-44}$, carp $GHRH\text{-}LIP_{1-2}$, and sGnRH were generously provided by W. Vale and J. Rivier (The Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, San Diego, CA 92037). Synthetic SRIF-14 was purchased commercially (Sigma Chemical Co., St. Loius, MO). For the in vivo experiments, the peptides were dissolved in saline immediately prior to injection. For each perifusion experiment, the human GHRH and the carp GHRH-LIP molecules were freshly dissolved in HBSS. sGnRH and SRIF-14 were dissolved in 0.01 N acetic acid and stored as concentrated (10 μg/20 μl) stock solutions at -25 °C. Several concentrations of each peptide were made immediately prior to the start of each perifusion experiment. The peptide solutions were kept at 4 °C before use but were allowed to equlibrate to 17 °C prior to administration.

Growth Hormone Measurement and Statistical Analysis

The GH content in serum samples from in vivo Experiments 1 and 2 and in perifusate samples from Experiment A was determined using the carp GH radioimmunoassay (RIA) described by Cook et al. [1983]. GH levels in serum samples from Experiment 3 and perifusate samples from Experiment B were measured using the carp GH RIA described in Appendix 1.

In Experiment 1, serum GH levels in groups of fish receiving $hGHRH_{1-40}$ or SRIF-14 were compared at each sample time to GH levels in the saline-injected group using the Mann-Whitney U-test ($p<0.05$) [Snedecor and Cochran, 1980]. In Experiments 2 and 3, serum GH levels at each sample time in the groups receiving injections of the peptides were compared to the levels in the saline-injected groups using Student's t-test ($p<0.05$) on logarithmically transformed data [Snedecor and Cochran, 1980].

7.3 RESULTS

In Vivo Experiments

In Experiment 1, groups of fish maintained at 20 °C receiving either 0.1 or 1.0 μg/g $hGHRH_{1-40}$ had signficantly elevated serum GH levels compared to the saline-injected group at 1 hour post-injection (Figure 7.1). At 20 °C, fish receiving SRIF-14 (1.0 μg/g) had significantly lower serum GH levels at 1 hour post-injection compared to the saline-injected group (Figure 7.1). At 3 hours after injection, serum GH levels were similar in all groups of fish kept at 20 °C. In fish maintained at 12 °C, serum GH levels in fish receiving either dosage of $hGHRH_{1-40}$ were similar to levels in the saline-injected group at both 1 and 3 hours post-injection (Figure 7.1). In the group at 12 °C receiving SRIF-14, serum GH levels were significantly lower at 1 and 3 hours after injection compared to the levels in the saline-injected group (Figure 7.1).

In Experiment 2, serum GH levels at the 1 hour sample time in groups receiving either 0.01 or 0.1 μg/g $hGHRH_{1-40}$ were similar to the levels in the saline-injected groups (Figure 7.2). However, at this sample time, serum GH levels in the group injected with 1.0 μg/g $hGHRH_{1-40}$ were significantly elevated compared to the saline-injected group (Figure 7.2). At 3 and 6 hours following injection, serum GH levels were similar in all groups (Figure 7.2).

In Experiment 3, fish receiving either sGnRH (0.1 μg/g) or $hGHRH_{1-44}$ (1.0 μg/g) had significantly elevated serum GH levels compared to the levels in the saline-treated group at 1 hour following injection

(Figure 7.3). At 3 hours post-injection, serum GH levels in the groups receiving saline or $hGHRH_{1-44}$ were similar. However, serum GH levels in the group receiving sGnRH were significantly higher than the levels in saline-treated fish at this sample time (Figure 7.3). At 8 hours after injection, serum GH levels were similar in all groups.

In Vitro Experiments

Exposure of goldfish pituitary fragments for 20 minutes to each concentration of $hGHRH_{1-40}$ or $hGHRH_{1-44}$ did not alter GH secretion from the pituitary fragments (Figure 7.4). GH levels in the fractions of perifusate collected during exposure of the various concentrations of the human GHRH peptides were similar to the GH levels in fractions collected before and after exposure of the fragments to the peptide solutions.

Administration of 5 minute pulses of various concentrations of $hGHRH_{1-44}$ or hGHRH-A did not alter GH release from goldfish pituitary fragments (Figure 7.5); GH levels in fractions collected during and immediately following exposure of fragments to these peptide solutions were similar to the GH levels measured in fractions collected before exposure. Administration of 5 minute pulses of various concentrations of carp $GHRH\text{-}LIP_{1-29}$, carp $GHRH\text{-}LIP_{1-44}$ or carp $GHRH\text{-}LIP_{1-45}$ also did not influence GH secretion from goldfish pituitary fragments (Figure 7.6). However, a 2 minute pulse of 50 nM sGnRH administered 45 minutes after the last pulse of the GHRH-like molecule resulted in the rapid stimulation of GH secretion from the pituitary fragments in all of the perifusion columns (Figures 7.5 and 7.6).

7.4 DISCUSSION

Although there have been a large number of studies demonstrating that mammalian GHRH stimulates GH secretion in mammals and birds (see Introduction), there has only been one report [Peter et al., 1984] on the influence of mammalian GHRH on GH release in a teleost species. In this study [Peter et al., 1984], ip injection of $hGHRH_{1-40}$ was found to elevate circulating GH levels in the goldfish. In the present study, ip injection of $hGHRH_{1-40}$ as well as $hGHRH_{1-44}$ also resulted in elevated serum GH levels in the goldfish, confirming the findings of Peter et al. [1984]. However, the response to the human GHRH peptides in the present study was somewhat variable. For example, in Experiment 1, $hGHRH_{1-40}$ at both dosages was effective in elevating serum GH levels in fish maintained at 20 °C but not 12 °C. Although this may indicate that water temperature influences the response to $hGHRH_{1-40}$ in the goldfish, Peter et al. [1984] found that similar concentrations of $hGHRH_{1-40}$ were effective at 12 °C. It is unlikely that this discrepancy represents seasonal variations in the responsiveness to $hGHRH_{1-40}$ as both studies were conducted at a similar time of year (November). The finding that SRIF-14 suppressed GH levels in fish maintained at both 12 and 20 °C in this experiment indicates that goldfish at both temperatures were responsive to hypothalamic peptides. Finally, in other experiments conducted at 12 °C in the present study, the human GHRH peptides at 1.0 μg/g were effective in increasing serum GH levels in the goldfish. Therefore, the influence of the human GHRH molecules on serum GH levels in the goldfish appears to be reproducible, although the response is variable between experiments.

Various GnRH molecules were found to elevate serum GH levels in the goldfish [Chapter 4], and the influence of sGnRH was compared to the effect of $hGHRH_{1-44}$ on serum GH levels in Experiment 3. At 1 hour following ip injection, both sGnRH and $hGHRH_{1-44}$ resulted in similarly elevated serum GH levels. However, at the 3 hour sample time, serum GH levels were elevated only in fish receiving sGnRH. This finding suggests the effect of sGnRH on serum GH levels in the goldfish is of longer duration than the influence of $hGHRH_{1-44}$.

In mammalian species, the GHRH peptides elevate circulating GH levels by acting directly on the pituitary to increase the secretion of GH [Frohman and Jansson, 1986; Gelato and Merriam, 1986]. However, results from *in vitro* experiments in the present study provide evidence suggesting that exposure of the goldfish pituitary to either $hGHRH_{1-40}$ or $hGHRH_{1-44}$ does not alter GH secretion *in vitro*. The lack of any effect of the human GHRH molecules on GH secretion *in vitro* was consistent between experiments in the present study. Exposure of freshly prepared fragments of the goldfish pars distalis for 20 minutes to concentrations up to 100 nM of $hGHRH_{1-40}$ or $hGHRH_{1-44}$ did not result in any alteration to the secretion of GH (Figure 7.4). Similarly, discrete 5 minute pulses of 10 to 1000 nM concentrations of $hGHRH_{1-44}$ at 30 minute intevals did not alter GH secretion in other experiments using pituitary fragments allowed to equilibrate overnight (Figure 7.5). In these latter experiments, however, a 2 minute pulse of sGnRH was effective in stimulating GH secretion, ruling out the possibility that the fragments were unresponsive to stimulation in these experiments. These results using perifused fragments of the goldfish pituitary are in contrast to the situation in mammals where concentrations of GHRH as low as 10 pM induces GH secretion *in vitro* [Spiess *et al*., 1983].

Exposure of goldfish pituitary fragments to hGHRH-A, also did not influence GH secretion. This analog consists of the first 29 amino acids of hGHRH$_{1-44}$, with a Nle replacing the methionine residue in position 27. Studies in mammals have shown that analogs containing the first 29 amino acids of hGHRH$_{1-44}$ retain full biological activity, although oxidation of the methionine residue in position 27 results in very reduced potency of the molecule [Frohman and Jansson, 1986; Robberecht *et al*., 1986]. Replacement with Nle prevents oxidation of the methionine and the subsequent loss of bioactivity. It is unlikely that oxidation of the methionine residue in hGHRH$_{1-40}$ or hGHRH$_{1-44}$ resulted in the loss of the biological activity of these molecules in the present study, as protection of this residue in hGHRH-A did not result in increased potency in terms of stimulating GH secretion from the goldfish pituitary *in vitro*.

The absence of any influence of the human GHRH peptides on GH secretion *in vitro* in the present study, even at relatively high concentrations (i.e. 1000 nM) and under conditions in which sGnRH stimulates GH secretion, argues strongly against a direct action of the human GHRH peptides on the goldfish pituitary to alter GH secretion. It is difficult, therefore, to explain the *in vivo* stimulatory effects of hGHRH$_{1-40}$ and hGHRH$_{1-44}$ on GH release in the goldfish. It is possible that the putative teleost hypothalamic GHRH molecule is structurally quite different from mammalian GHRH, and that receptors on the goldfish pituitary are unable to recognize mammalian forms of the GHRH molecule at the concentrations tested in the present study. Alternatively, it is also possible that the human GHRH peptides alter circulating GH levels through an action(s) on tissue(s) other that the pituitary. A few studies in mammalian species have reported effects of mammalian GHRH molecules on several peripheral tissues [Pandal *et* *al*., 1984; Hauner *et* *al*., 1985], including the stimulation of endocrine hormone secretion from the isolated, perfused pancreas of the dog [Hermanssen *et* *al*., 1986]. Although similar results from studies in teleost species are not available, the possibility of peripheral effects of the human GHRH peptides resulting in the elevation of serum GH levels in the goldfish cannot be eliminated.

Three forms of a molecule with partial structural homology to mammalian GHRH were recently isolated from the hypothalamus of the carp [Vaughan *et* *al*., 1987; J. Rivier and W. Vale, personal communication], and it was possible that this molecule may represent a teleost GH-releasing factor. However, all three forms of carp GHRH-LIP, the 45 amino acid peptide as well as the 29 and 44 amino acid derivatives, were ineffective in altering GH secretion from goldfish pituitary fragments in the present study. The carp GHRH-LIP molecules were isolated based on their immunological properties, rather than their biological actions, using an antiserum generated against rat GHRH [Vaughan *et* *al*., 1987]. Furthermore, the carp GHRH-LIPs have only partial structural homology to mammalian GHRH, and Vaughan *et* *al*. [1987] indicated that the carp GHRH-LIP is actually more closely related structurally to the family of mammalian peptides that includes vasoactive intestinal polypeptide and PHI/PHM.

The physiological action of the GHRH-LIPs isolated from the carp hypothalamus remains unknown, although results from the present study indicate that this peptide probably does not directly influence GH secretion from the pituitary. This does not rule out the possibility that other GHRH-like peptides stimulating GH secretion from the pituitary may be present in the hypothalamus of teleost fishes. Additional studies are required to fully characterize the physiological effects of mammalian GHRH peptides in goldfish and other teleost species, and to fully examine the nature of the substances in the teleost hypothalamus that stimulate pituitary GH secretion. Until this information is available, however, a role for a GH-releasing factor similar in structure to mammalian GHRH in the neuroendocrine regulation of GH secretion in the goldfish and other teleost species remains questionable.

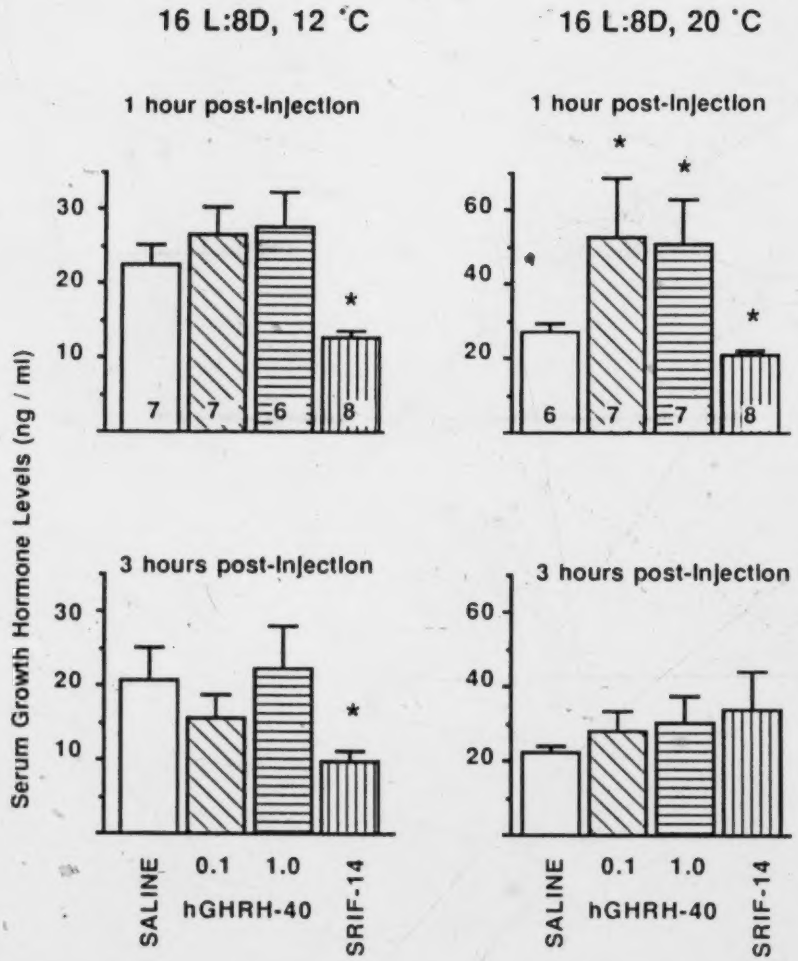

Figure 7.1. Influence of a single intraperitoneal injection of hGHRH$_{1-40}$ (0.1 and 1.0 µg/g) or SRIF-14 (1.0 µg/g) on serum growth hormone (GH) levels at 1 (top panels) and 3 (bottom panels) hours after injection in female goldfish held at either 12 °C (left panels) or 20 °C (right panels) in November. All values are represented as mean ± SEM. Sample sizes are indicated along the abscissa in each panel. Significant ($p<0.05$) differences in serum GH levels at each sample time between saline-treated group and the groups receiving the peptides are indicated by an asterisk.

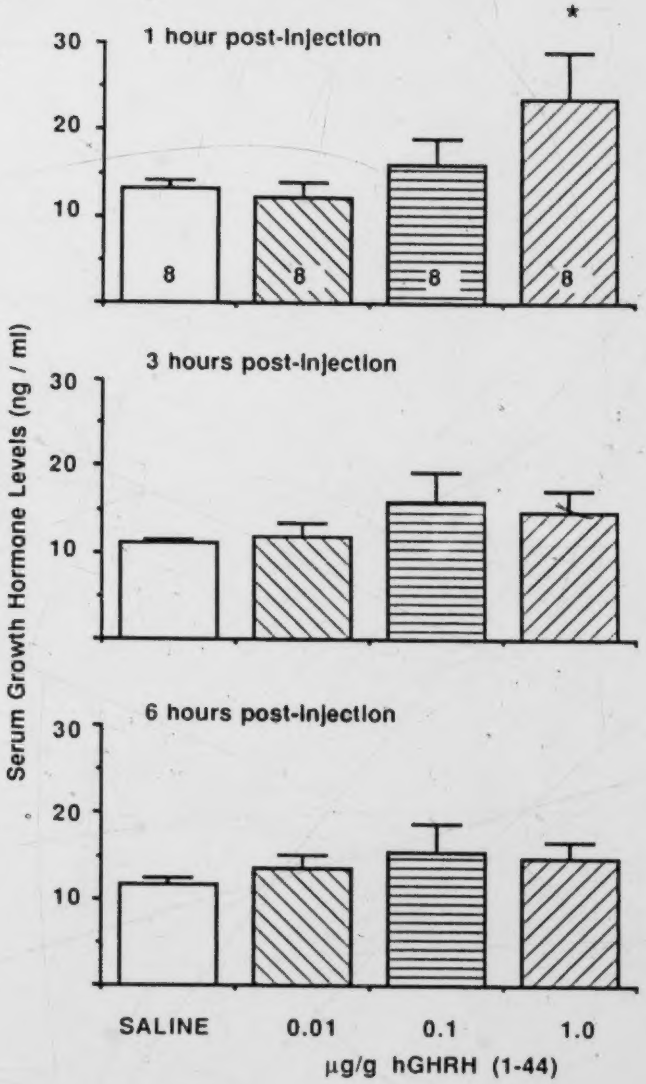

Figure 7.2. Influence of a single intraperitoneal injection of three concentrations of $hGHRH_{1-40}$ (0.01, 0.1 and 1.0 µg/g) on serum growth hormone levels at 1, 3 and 6 hours after injection in female goldfish held at 12 °C in May. All values are represented as mean ± SEM. Sample sizes are indicated along the abscissa in the lower panel. Significant ($p<0.05$) differences in serum GH levels at each sample time between saline-treated group and the groups receiving the peptides are indicated by an asterisk.

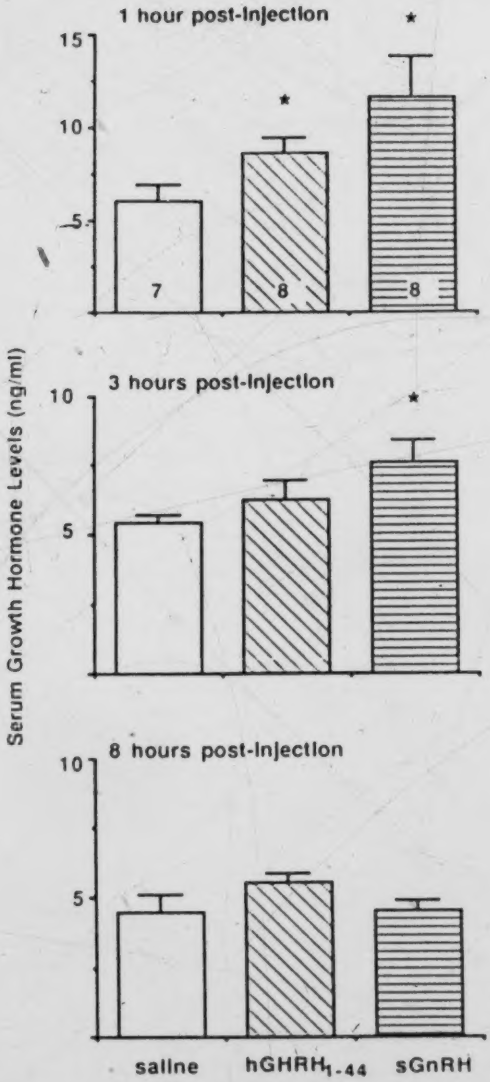

Figure 7.3. Influence of a single ip injection of $hGHRH_{1-44}$ (1.0 µg/g) or sGnRH (0.1 µg/g) on serum GH levels in female goldfish held at 12 °C in April. Sample sizes in each group are indicated along the abscissa in the bottom panel; all values are represented as mean ± SEM. Significant ($p<0.05$) differences in serum GH levels at each sample time between the saline-injected group and the groups receiving the peptides are indicated by an asterisk.

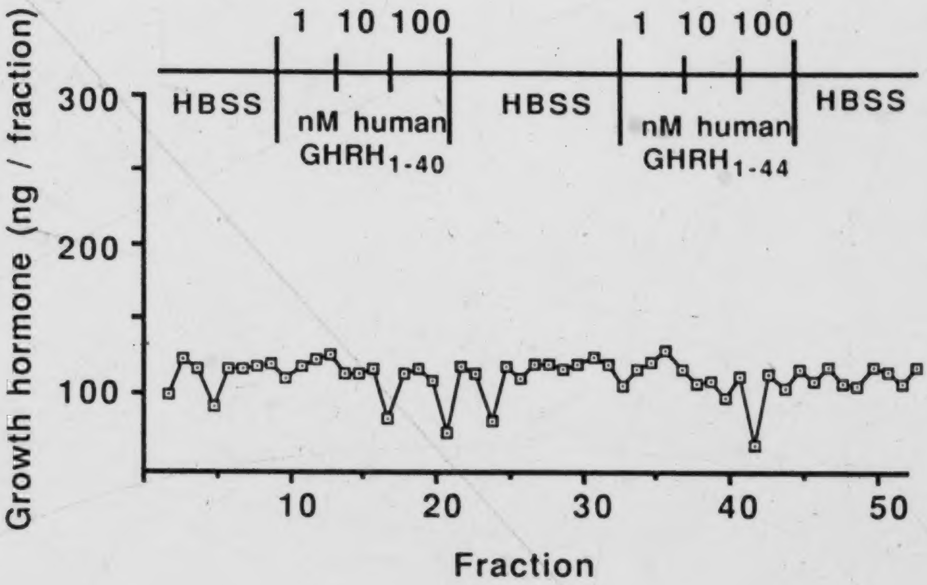

Figure 7.4. Representative profile of GH levels in fractions (1.25 ml) of perifusate collected from an individual perifusion column, comparing the influence of exposure to various concentrations of $hGHRH_{1-40}$ and $hGHRH_{1-44}$ on GH secretion from fragments of the goldfish pars distalis. The treatment protocol is shown along the top of the graph. The results presented here were similar to results obtained in other perifusion columns.

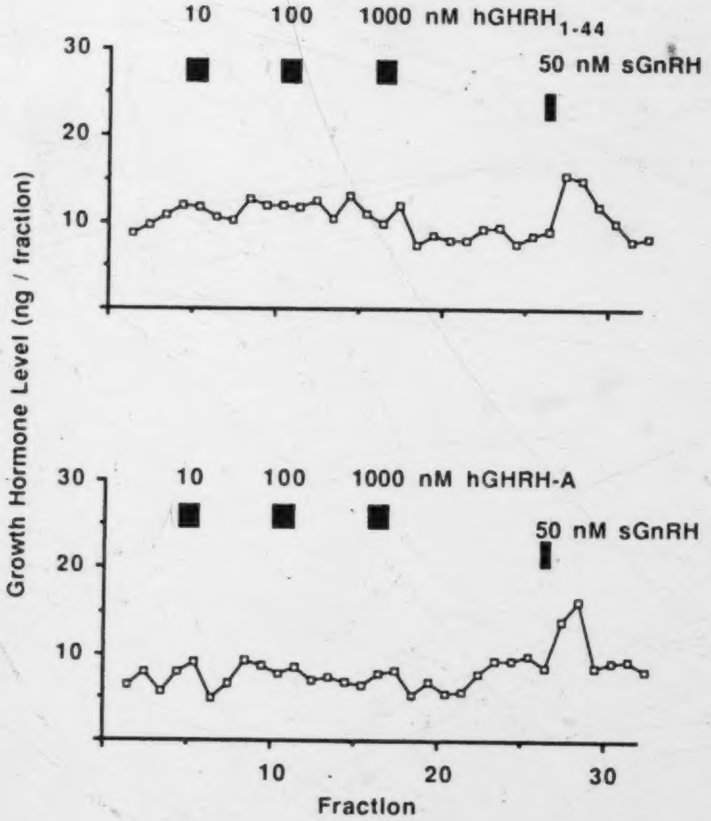

Figure 7.5. Representative profiles of GH levels in fractions of perifusate collected from individual perifusion columns containing fragments of the goldfish pituitary which were exposed to 5 minute pulses (at 30 minute intervals) of various concentrations of hGHRH$_{1-44}$ (top panel) and hGHRH-A (bottom panel). The fragments in each column were also exposed to a 2 minute pulse of 50 nM sGnRH 45 minutes after the last pulse of the hGHRH peptide. The pulses of peptides are represented by the black bars at the top of each panel. Results similar to those presented here were obtained using fragments in at least one other perifusion column.

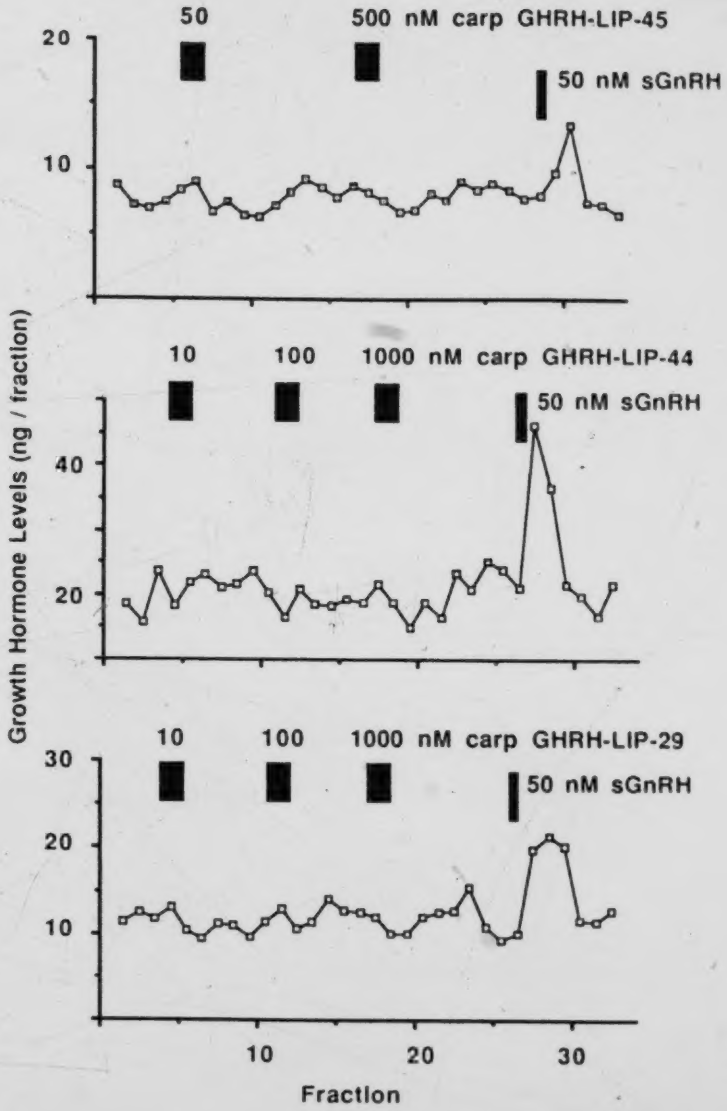

Figure 7.6. Representative profiles of GH levels in fractions of perifusate collected from individual perifusion columns containing fragments of the goldfish pituitary which were exposed to 5 minute pulses of various concentrations of carp GHRH-LIP$_{1-45}$ (top panel), carp GHRH-LIP$_{1-44}$ (middle panel) or carp GHRH-LIP$_{1-29}$ (bottom panel). The fragments in each column were also exposed to a 2 minute pulse of 50 nM sGnRH following the last pulse of the carp GHRH-LIP. The pulses of peptides are represented by the black bars at the top of each panel. The results presented here were similar to results obtained in at least one other perifusion experiment.

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8. GENERAL DISCUSSION

Current understanding of the hypothalamic regulation of GH secretion in vertebrates has come primarily from studies in mammalian and, to a lesser extent, avian species [for review: Chapter 1]. The role of the hypothalamus in controlling GH secretion in poikilothermic vertebrates has not been extensively studied. Consequently, the control of GH secretion by the central nervous system (CNS) is poorly understood in teleost fishes and other lower vertebrates. In the preceding chapters of the present thesis, new and original information regarding the hypothalamic regulation of GH secretion in the goldfish was presented. This final chapter will briefly review and discuss the major findings of the present thesis, and will propose a model of the hypothalamic regulation of GH secretion in the goldfish.

The influence of various somatostatin (SRIF) molecules on GH release from perifused fragments of the goldfish pituitary was studied in Chapter 2. Previously, the tetradecapeptide, SRIF-14, had been isolated from teleost tissues [Andrews and Dixon, 1981; Noe *et al.*, 1979; Plisetskaya *et al.*, 1986], and was shown to decrease circulating GH levels in the goldfish [Cook and Peter, 1984]. In the present study, SRIF-14 was found to rapidly inhibit GH secretion *in vitro* [Chapter 2]. This demonstrates that SRIF-14 acts directly at the level of the pituitary to alter GH release in the goldfish, and provides support for the hypothesis that SRIF-14 or a very similar molecule functions as a GH release inhibitory factor in teleosts.

Previous studies in the tilapia *Orechromis mossambicus* [Fryer *et al*., 1979; Rivas *et al*., 1986] and *Poecilia latipinna* [Wigham and Batten, 1984] have also found that SRIF-14 inhibits GH secretion *in vitro*. However, relatively high concentrations (30 to 1000 nM) were used in these studies [Fryer *et al*., 1979; Rivas *et al*., 1986; Wigham and Batten, 1984], and the present study in the goldfish demonstrates that much lower concentrations of SRIF-14 are effective in altering GH release in a teleost species [Chapter 2]. For example, the half maximal effective dose (ED$_{50}$) of SRIF-14 determined following exposure of goldfish pituitary fragments to 2 minute pulses of various concentrations of SRIF-14 was calculated to be 1.3 nM (Figure 2.4), very similar to the ED$_{50}$ of SRIF-14 on the mammalian pituitary [Vale *et al*., 1975]. This represents the first demonstration that physiological concentrations of SRIF-14 inhibit GH secretion in a teleost species, and that the potency of SRIF-14 in at least one teleost species is similar to the potency of SRIF-14 on GH secretion in other vertebrates.

The present study is the first to examine the influence of SRIF peptides other than SRIF-14 on GH release in a teleost species [Chapter 2]. Mammalian SRIF-28 was found to be equipotent with SRIF-14 in inhibiting GH release from the goldfish pituitary *in vitro* (Figure 2.5). In mammals, SRIF-14 and SRIF-28 are derived from the same precursor molecule, and the carboxyl terminus of mammalian SRIF-28 contains the complete sequence of SRIF-14 [Goodman *et al*., 1982; Shen *et al*., 1982]. Therefore, it is possible that the equipotency of these two peptides in the present study is due to this structural homology between SRIF-14 and SRIF-28. Although it is not known if the teleost precursor for SRIF-14 (proSRIF-I) is also processed to a longer peptide corresponding to mammalian SRIF-28, results from the present study in the goldfish indicate that peptides containing the carboxyl terminus sequence of SRIF-14 may also be biologically active in teleosts.

Teleosts appear to be unique among vertebrates in that two genes encoding two distinct SRIF precursor molecules (proSRIF-I and proSRIF-II) are present in pancreatic islet tissue [for review: Chapter 2]. In all teleost species examined to date, SRIF-14 is produced from proSRIF-I, whereas the SRIF molecule produced from proSRIF-II is structurally different from SRIF-14 and also displays some species difference in structure. In the present study, the effects of SRIF molecules isolated from islet tissue of channel catfish and coho salmon were compared to the influence of SRIF-14 on GH secretion from the goldfish pituitary in vitro. Both catfish SRIF-22 (cSRIF-22) and salmon SRIF-25 (sSRIF-25) did not influence the release of GH from perifused goldfish pituitary fragments, although equivalent dosages of SRIF-14 were effective in the same experiments (Figures 2.5 and 2.6). This finding indicates that the SRIF receptors in the goldfish pituitary are specific for molecules containing the SRIF-14 sequence, and that slight alterations to the SRIF molecule, especially in the carboxyl terminus region, result in the loss of biological activity in terms of the inhibition of GH secretion. Furthermore, this suggests that the SRIF peptide derived from the second SRIF gene in teleosts may not participate directly in the regulation of GH secretion from the teleost pituitary.

Based on the observation that the two forms of SRIF in the teleost pancreas are structurally dissimilar, it has been suggested that the various SRIF molecules may have quite different biological functions in teleosts [for review: Chapter 2]. In support of this hypothesis, there does appear to be some variation in the expression of the two SRIF genes in various teleost tissues. An immunocytochemical study has demonstrated that the genes encoding for proSRIF-I and proSRIF-II, and, consequently, the cleavage products from each gene (SRIF-14 and anglerfish SRIF-28, respectively) are expressed in different D-cells within the anglerfish pancreas [McDonald et al., 1987]. Furthermore, a study using specific radioimmunoassays to measure the relative amounts of SRIF-14 and cSRIF-22 in various tissues in the channel catfish [Fletcher et al., 1983], found that the predominant immunoreactive form of SRIF in the catfish brain corresponded to SRIF-14, whereas the predominant form in the pancreas was cSRIF-22. Thus, it is possible the biological actions of the products of the two SRIF genes may be related to their differential expresssion in various tissues. Although additional studies are required to fully characterize the complete range of physiological actions of the two SRIF molecules in teleosts, results of the present study suggest that at least one biological action, the inhibition of GH secretion from the pituitary, may not be shared by SRIF-14 and the product of the second teleost SRIF gene.

Although immunocytochemical studies have found cell bodies and nerve fibers containing immunoreactive SRIF (irSRIF) widely distributed throughout the teleost CNS [for review: Chapter 3], the present study is the first to examine the relationship between circulating GH levels and endogenous brain and pituitary irSRIF in a teleost species. In Chapter 3, it was found that the amount of irSRIF in extracts of the pituitary, and the telencephalic and hypothalamic regions of the goldfish forebrain at various times of the year was inversely related to seasonal changes in circulating GH levels (Table 3.1). The amount of irSRIF in the forebrain regions and the pituitary was highest at the time of year when serum GH levels were the lowest (November and February), and, conversely, lowest at the time of year when serum GH levels were the highest (May, June and July). This provides the possibility that changes in the irSRIF content in the goldfish forebrain and pituitary may be involved in determining the seasonal pattern in circulating GH levels in the goldfish. Interestingly, results presented in Chapter 2 indicate that the goldfish pituitary in vitro is most responsive to SRIF-14 in February, a time of year corresponding to a high pituitary irSRIF content and low serum GH levels. This finding suggests that the responsiveness of the goldfish pituitary to SRIF-14 may also vary seasonally in a positive relationship to the endogenous irSRIF content.

Additional evidence presented in Chapter 3 also supports the role of the forebrain in the regulation of GH secretion in the goldfish. In a brain lesioning experiment, destruction of the preoptic area of the goldfish forebrain resulted in increased serum GH levels, concommitant with a decrease in the amount of irSRIF in the pituitary (Figure 3.3). This provides direct evidence that the preoptic region is the origin of a somatostatinergic projection to the goldfish pituitary. Furthermore, these results provide support for a functional relationship between endogenous irSRIF in the CNS and GH secretion in the goldfish, and for the hypothesis that a SRIF-14-like molecule functions as a GH release-inhibitory factor in teleosts.

A novel finding of the present thesis is the demonstration that gonadotropin (GTH)-releasing hormone (GnRH) stimulates GH secretion in the goldfish. In Chapter 4, it was found that intraperitoneal (ip) injection of various GnRH peptides, including native salmon GnRH (sGnRH), resulted in elevated circulating levels of GH in both female and male goldfish. *In vitro* experiments using perifused fragments of the goldfish pituitary [Chapter 5] provide evidence that the effect of GnRH on GH secretion occurs directly at the level of the pituitary. In a dose-response experiment, sGnRH was found to rapidly stimulate the secretion of both GH and GTH *in vitro* in a dose-dependent manner (Figure 5.5). Analysis of the dose-response curves obtained from the *in vitro* experiments (Figure 5.6) indicates an ED$_{50}$ of sGnRH in the physiological nM range, and was similar for both GH and GTH secretion. The time course of stimulation of GH secretion *in vitro* by sGnRH was very similar to the time course of GTH stimulation (Figure 5.5). Furthermore, exposure of pituitary fragments to the analog [D-Arg^6,Pro^{9}NEt]-sGnRH (sGnRH-A) resulted in the rapid desensitization of the goldfish pituitary, in terms of both GH and GTH release, to further stimulation by GnRH (Figures 5.2 and 5.3). These results provide evidence that GnRH stimulates the secretion of both GH and GTH from the goldfish pituitary, and suggests that the receptor mechanisms mediating the stimulatory actions of GnRH may be similar for both GH and GTH secretion.

Although GnRH can stimulate the release of both GH and GTH from the goldfish pituitary, an important finding in the present thesis is the observation that the secretion of both hormones can occur independent of each other. For example, ip injection of pimozide, a dopamine antagonist, greatly potentiates the GnRH stimulation of GTH secretion in the goldfish and other teleost species through the removal of the endogenous inhibitory influence of dopamine on GTH secretion [Peter *et al.*, 1986]. In contrast, ip injection of pimozide does not potentiate GnRH-induced GH secretion in either female (Table 4.1) or male (Figure 4.3) goldfish, indicating that dopamine does not inhibit the GnRH stimulation of GH release. The independent release of GTH and GH was also observed in vitro [Chapter 5]. In this experiment, SRIF-14 was found to completely inhibit the GnRH-induced secretion of GH, without influencing the GnRH stimulation of GTH release (Figure 5.7). Conversely, apomorphine, a dopamine agonist, completely abolished GnRH-induced GTH secretion, but did not alter the GnRH stimulation of GH secretion. These results provide strong evidence that the stimulation of GH release can occur independently of stimulated GTH release, and also provides evidence that the release inhibitory factors influencing GH and GTH secretion in the goldfish are separate and distinct.

In the rat, recent studies using reaggregates of enriched populations of lactotrophs and gonadotrophs have found that mammalian GnRH (mGnRH) stimulates prolactin (PRL) release through a mechanism that is dependent on the presence of gonadotrophs, and it is hypothesized that gonadotrophs in the rat pituitary secrete a factor which stimulates PRL release through a paracrine action [Denef et al., 1986]. Although paracrine interactions in the goldfish pituitary have not been studied, the finding that the GnRH stimulation of GH release can occur independent of stimulated GTH release suggests that the action of sGnRH on GH secretion is not likely due to a paracrine influence of the gonadotrophs on the somatotrophs. For similar reasons, it is also unlikely that the GnRH-induced secretion of GTH is due to a paracrine effect of the somatotrophs on the gonadotrophs.

In the goldfish pituitary, the somatotrophs and the gonadotrophs are found intermingled in close proximity to each other in the proximal pars distalis [Cook *et al*., 1983; Kaul and Vollrath, 1974]. Immunocytochemical studies have found that neuronal fibers containing immunoreactive GnRH (irGnRH) penetrate into the proximal pars distalis of the goldfish [Kah *et al*., 1984; Kah *et al*., 1986]. Direct contacts between the neuronal fibers containing irGnRH and somatotroph cells have yet to be reported in the goldfish or other teleosts, but the neuronal fibers containing irGnRH within the proximal pars distalis are certainly in the vicinity of the somatotrophs, as well as the gonadotrophs. This provides the possibility of an anatomical relationship between somatotrophs and irGnRH fibers in the goldfish, although additional ultrastructural studies are required to provide firm evidence of the innervation of the somatotrophs by GnRH-containing neuronal fibers.

In Chapter 4, it was found that a single ip injection of the analog [D-Ala^6,Pro^{9}NEt]-mGnRH (mGnRH-A) resulted in elevated serum GH levels in female goldfish for up to 24 hours (Table 4.1). The serum levels of GH measured following injection of mGnRH-A were similar to levels measured previously in goldfish displaying rapid rates of body growth [Marchant and Peter, 1986; Marchant *et al*., 1986]. This provided the possibility that the increases in serum GH levels induced by mGnRH-A are sufficient to accelerate body growth in the goldfish. This hypothesis was tested by measuring body growth rates in goldfish receiving repeated injections of mGnRH-A [Chapter 6]. In all three experiments [Chapter 6], the injections of mGnRH-A resulted in significant increases in body length in goldfish held at 12 °C, a water temperature suboptimal for growth in the goldfish [Marchant *et al*., 1986]. Coinjection of an antiserum raised against carp GH partially blocked the growth response to mGnRH-A, whereas an antiserum against carp GTH did not influence growth induced by mGnRH-A. This suggests that the increases in body growth rate in the goldfish measured following injection of mGnRH-A are probably due to increases in serum GH levels, and indicates that the increase in serum GH levels following mGnRH-A injection in the goldfish are sufficient to accelerate body growth.

Results presented in Chapter 7 indicate that ip injection of another mammalian hypothalamic peptide, human GH-releasing hormone (GHRH), elevates serum GH levels in goldfish, confirming the previous findings of Peter *et al*. [1984]. However, in experiments using perifused pituitary fragments, various GHRH peptides did not alter the secretion of GH from the goldfish pituitary [Chapter 7]. Furthermore, a teleost GHRH-like peptide (carp GHRH-LIP) isolated from hypothalami of the commmon carp, was also ineffective in altering GH release *in vitro*. These *in vitro* results suggest that the influence of GHRH on GH secretion *in vivo* in the goldfish is probably not due to an influence of GHRH directly at the level of the pituitary, although the manner by which GHRH acts to alter GH secretion *in vivo* in the goldfish remains unknown. Consequently, the involvement of a GHRH-like peptide in the hypothalamic regulation of GH secretion in the goldfish remains questionable.

In conclusion, the major findings of the present thesis have been incorporated into a hypothetical model of the hypothalamic regulation of GH secretion in the goldfish (Figure 8.1). This model does not rule out the possibility that other substances (e.g. catecholamines or other hypothalamic peptides) may also influence GH secretion in the goldfish. However, based on results from experiments in the present study, it appears that the secretion of both GH and GTH are regulated, at least in part, through a common releasing factor, GnRH. Previous studies have demonstrated that dopamine functions as a GTH release-inhibitory factor in teleosts [Peter _et_ _al_., 1986], whereas the present study provides firm evidence that SRIF-14 or a very similar molecule functions as GH release-inhibitory factor. Therefore, the release-inhibitory factors for GH and GTH secretion in the goldfish appear to be separate and distinct. This model provides a firm foundation for future studies investigating the hypothalamic regulation of GH secretion in the goldfish and other teleost species.

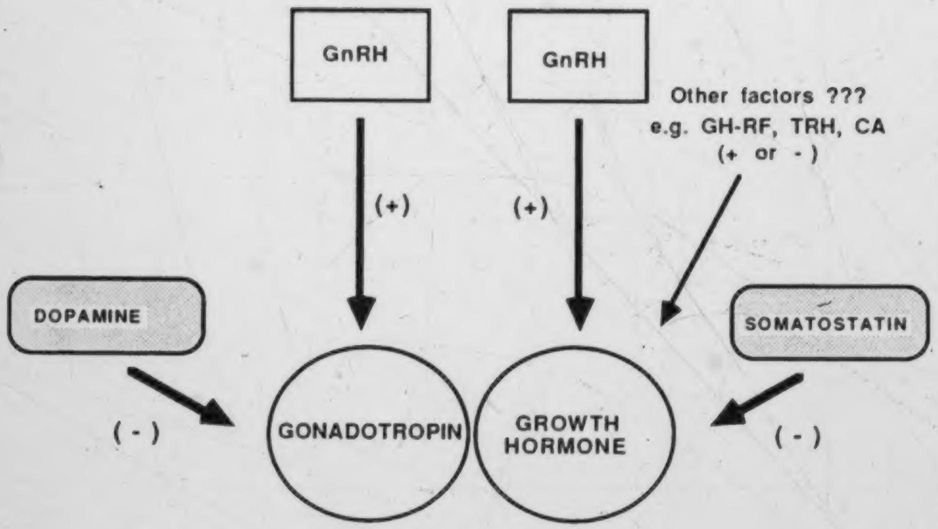
GnRH
GnRH
Other factors ???
e.g. GH-RF, TRH, CA
(+ or -)
(+)
(+)
DOPAMINE
SOMATOSTATIN
(-)
GONADOTROPIN
GROWTH HORMONE
(-)

Figure 8.1. Proposed model of the hypothalamic regulation of growth hormone and gonadotropin secretion in the goldfish. See text for a detailed description of this model. Abbreviations: GH-RF - growth hormone-releasing factor; TRH - thyrotropin-releasing hormone; CA - catecholamines.

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9. APPENDIX I

CARP GROWTH HORMONE RADIOIMMUNOASSAY

Growth hormone (GH) was purified from pituitaries of the common carp (*Cyprinus carpio* L.) by H. Kawauchi and coworkers (School of Fisheries Sciences, Kitasato University, Sanriku, Iwate 022-01, Japan). A detailed description of the purification procedures and biochemical characteristics of the carp GH preparation will be published elsewhere. The carp GH preparation is biologically active: weekly injections of the carp GH cause a dose-dependent increase in body weight and length in young carp (Kawauchi *et al*. unpublished results). This carp GH preparation was used to develop a specific radioimmunoassay (RIA) suitable for measuring pituitary and circulating levels of GH in the goldfish.

Generation of Antiserum Against Carp GH

Antiserum to the purified carp GH was generated in a young male rabbit (Flemish giant X French lop-earred hybrid). The rabbit received a sequence of injections, at 14 day intervals, of 200, 100 and 100 µg carp GH (dissolved in 1 ml of 0.6 % NaCl and 1 ml of Freund's complete adjuvant) at multiple subcutaneous sites and 1 intramuscular site. Following the third injection of carp GH plus Freund's complete adjuvant, two booster injections of 50 µg carp GH in 1 ml 0.6 % NaCl were given at 14 day intervals. Eight days after the last booster injection, the rabbit was completely bled by cardiac puncture. The blood was allowed to clot for several hours at 4 °C, and the resulting serum was collected, frozen and lyophilized.

The ability of the antiserum to neutralize endogenous circulating GH in goldfish was assessed by monitoring growth rates following intraperitoneal (ip) injection of the antiserum. For this experiment, female goldfish were fin-clipped for individual identification and held at 16L:8D, 18 °C. The average body weight of the fish at the start of the experiment was 7.5 ± 0.3 g (n=20), whereas the average body length (as measured from the tip of the snout to the end of the caudal peduncle) was 8.1 ± 0.08 cm. During the pre-treatment period, all fish received 4 ip injections at 3 day intervals of 50 µl of teleost physiological saline [Burnstock, 1958] supplemented with Penicillin-G (260 U/ml) and gentamycin sulphate (0.2 mg/ml). During the treatment period, groups of goldfish received 4 ip injections of 50 µl of normal rabbit serum (NRS treated; n=11) or 50 µl of the rabbit anti-carp GH serum (racGH treated; n=9) at 3 day intervals. During the post-treatment period, all fish received 4 injections of 50 µl of the saline solution at 3 day intervals. Total body weight and length were measured at 12 day intervals throughout the experiment. Growth rates during the pre-treatment, treatment and post-treatment periods were calculated as the % increase in weight or length over each 12 day period. Growth rates of the the NRS and racGH treated groups were compared using Student's t-test on logarithmic transformed data.

During the pre-treatment period, both groups of goldfish displayed similar rates of growth (Figure 9.1). However, during the treatment period, the rate of increase in both weight and length was significantly lower in the racGH treated group compared to NRS treated fish. The rate of increase in body weight remained significantly lower in the racGH treated fish during the post-treatment period; the growth rate, based on changes in length, was also depressed in the racGH treated group during the post-treatment period, although the difference was not statistically significant (Figure 9.1). In the NRS treated group, the rate of change in weight showed a gradual decrease over the experimental period, possibly due to handing stresses associated with the experimental protocol. However, the rate of increase in length remained constant throughout the experimental period. Results of this experiment indicate that the antiserum generated against carp GH depressed the rate of body growth in goldfish, suggesting that endogenous GH was neutralized by the antiserum. This provides evidence that the antiserum generated against the carp GH recognizes a biologically active GH in the goldfish.

The immunocytochemical staining characteristic of the carp GH antiserum on the goldfish pituitary gland has also been examined [H. Cook and R. Peter, unpublished results]. The antiserum at a final dilution of 1:3000 reacted with only the somatotrophs of the anterior pituitary gland. Preabsorption of the antiserum with the purified carp GH, but not carp prolactin, completely abolished the staining reaction. These results provide further evidence of the specificity of the antiserum for endogenous GH in the goldfish.

Preparation of ^{125}I-carp GH

Carp GH was iodinated using procedures similar to those described previously [Cook *et al*., 1983]. One mCi of ^{125}I-Na in 50 µl of 0.5 M phosphate buffer (pH=7.4) was added to a conical vial containing 5 µg of carp GH in 5 µl of 0.05 M phosphate buffer (pH=7.4). Ten µl of lactoperoxidase (1 I.U.; Calbiochem, La Jolla, CA) was added, and the reaction was initiated by the addition of 10 µl of 0.003% H_2O_2. The reaction mixture was gently agitated, and 2 additional 10 µl aliquots of the H_2O_2 solution were added at 5 minute intervals. The reaction was terminated by dilution through the addition of 500 µl of 0.05 M phosphate buffer. The reaction mixture was fractionated by gel filtration on a 1 X 50 cm column of Sephadex G-100 fine (Pharmacia, Dorval, Que.). The elution buffer consisted of 0.08 M sodium barbital (pH=8.6) supplemented with 0.5 % bovine serum albumin (BSA). Prior to chromatography, the column was washed with 2 ml of barbital buffer containing 5 % BSA to prevent adsorption of the reaction mixture to the column. The void volume of the chromatography column was determined as the elution volumn of a 0.05 % solution of dextran blue. One ml fractions of the eluate were collected using an automatic fraction collector, and the radioactivity in each fraction was monitored.

Chromatography of the reaction mixture results in the separation of 2 major peaks of radioactivity (Figure 9.2a). At the start of the first peak there is a sharp increase in radioactivity in fractions corresponding to the void volume of the column (fractions 15-19), followed by a long shoulder of radioactivity (fractions 20-35). The second peak of radioactivity elutes in fractions 46 through 52. The position of ^{125}I-carp GH in this profile was determined in two ways: by determining the fractions exhibiting the highest specific binding of radioactivity to the antiserum generated against carp GH, and by determining the chromatographical profile of 'cold' carp GH under conditions identical to those used to fractionate the iodination reaction mixture. Specific binding of radioactivity to the antiserum was detected in fractions 16 through 40 (Figure 9.2a), with the highest specific binding occurring in the fraction corresponding to the shoulder of the first peak of radioactivity. Little or no specific binding was detected in fractions from the second peak of radioactivity (Figure 9.2a). The chromatographic profile of 'cold' carp GH was determined by fractionating 300 ng of the carp GH standard on a 1 X 50 cm column of Sephadex G-100, and measuring immunoreactive (ir) carp GH in 1 ml fractions of the eluate using the RIA described below. Immunoreactive carp GH was detected in fractions 16 through 32 (Figure 9.2b), with a small peak of ir carp GH in fractions corresponding to the void volume and a larger peak of ir carp GH occurring in fractions 24 through 29. This indicates that 'cold' carp GH elutes in a postion corresponding to the first peak of radioactivity detected after chromatography of the iodination reaction mixture. Notably, the largest peak of ir carp GH was detected in fractions (Figure 9.2b) corresponding to the region of radioactivity displaying the highest specific binding to the antiserum (Figure 9.2a). Consequently, fractions from this region of the chromatographic profile of the iodination reaction mixture were selected as containing the greatest amount of ir ^{125}I-carp GH, and were pooled for use in the RIA. The pooled fraction of ^{125}I-carp GH was stored at 4 °C, and was stable for up to 3 weeks under these conditions.

The specific activity of the ^{125}I-carp GH prepared in this manner was determined by incubating various amounts of the ^{125}I-carp GH with a constant amount of antiserum (final dilution 1:81,000), and comparing the displacement curve obtained in this way to the displacement curve obtained by incubating various amounts of carp GH standard with the antiserum in the presence of 10^4 cpm (0.0065 μCi) of ^{125}I-carp GH [Van Der Kraak, 1983]. The displacement curve produced by increasing concentrations of ^{125}I-carp GH is parallel to the displacement curve produced by the carp GH standard (Figure 9.3), with 0.065 μCi of ^{125}I-carp GH corresponding to 0.26 ng of the carp GH standard. Therefore, the specific activity of the ^{125}I-carp GH preparation was calculated as 248 μCi per μg of carp GH.

RIA Protocol

The RIA protocol is similar to that described previously [Cook *et al.*, 1983]. The assay buffer consisted of 0.08 M sodium barbital (pH=8.6) containing 0.5 % BSA. The RIA was performed using the antiserum at a final dilution of 1:117,000, and with approximately 10^4 cpm of ^{125}I-carp GH. Incubation tubes containing 100 μl of antiserum (initial dilution of 1:52,000 in assay buffer, and containing normal rabbit serum at a dilution of 1:40) and 25 μl of standard (0 to 100 ng/ml) or sample were incubated at 4 °C for 24 hours. Following this incubation, 100 μl of ^{125}I-carp GH appropriately diluted in the assay buffer was added to each incubation tube. After an additional 24 hour incubation at 4 °C, 200 μl of goat anti-rabbit serum (Calbiochem) diluted 1:20 in assay buffer was added, and the incubation continued for a final 24 hours. On the fourth day, the tubes were centrifuged, the supernatant decanted, and radioactivity in the pellets counted. Non-specific binding (NSB) of the radioacitivity was determined as the amount of radioactivity contained in pellets of tubes incubated in the absence of antiserum, and was similar to NSB measured in the presence of antiserum incubated with an excess of the standard (< 10 % of total radioactivity originally present). Specific binding of ^{125}I-carp GH to antiserum incubated in the absence of standard was corrected for NSB and represented approximately 23 % of the radioactivity originally present.

The minimum sensitivity of the RIA, defined as the minimum concentration of the standard resulting in significant (p=0.01) displacement of the specifically bound ^{125}I-carp GH from the antiserum [Reuter et al., 1978], was calculated as 0.625 ng GH/ml. The concentration of carp GH resulting in 50 % displacement of specifically bound ^{125}I-carp GH was calculated as 9.74 ± 0.69 ng/ml (mean ± SEM; n=7). Variability within the assay or between assays was determined by measuring ir carp GH in serum samples 3 times in a single assay or in 3 separate assays, respectively. The within assay variability for serum samples containing 4.43 and 55.1 ng ir GH/ml was calculated as a % coefficient of variation (% CV) of 7.7 and 6.4, respectively; the between assay variability for each sample was calculated as 8.5 and 7.5 % CV, respectively. The intra- and inter-assay variabilities were within acceptable limits, and were similar to variabilities described previously [Cook et al., 1983].

Assay Validation

The hormonal specificity of the antiserum was assessed by evaluating the crossreactivity of various vertebrate pituitary hormone preparations with the antiserum (Figure 9.4). Salmon (*Oncorhynchus keta*) GH [Kawauchi et al., 1986], bovine GH (NIH-GH-B18), carp prolactin [Yasuda et al., 1987], salmon prolactin [Yasuda et al., 1986], ovine prolactin (Sigma Chemical Co., St. Louis MO), and carp gonadotropin [Peter et al., 1984] at concentrations up to 500 ng/ml do not show any significant crossreactivity (<0.1 %) with the antiserum. In contrast, the displacement curve produced by a previously described preparation of carp GH [Cook et al., 1983] is parallel to that of the newly purified carp GH standard curve; the poteny of the carp GH preparation described by Cook _et_ _al_. [1983] in the present RIA was estimated by computer analysis [Pekary, 1979] as 45 % of the carp GH standard.

The suitability of the RIA for measuring GH levels in goldfish serum and homogenates of the goldfish pituitary gland was determined by comparing displacement curves produced by serial dilutions of goldfish serum and pituitary homogenates with the displacement curve produced by the carp GH standard. Serial dilutions from fish injected with saline or 0.1 µg/g of mGnRH-A produce displacement curves parallel to the carp GH standard (Figure 9.5). Notably, serum obtained 7 days post-operation from hypohysectomized goldfish resulted in displacement indistinguishable from the zero dose level, whereas serum from sham-operated goldfish resulted in displacement parallel to the carp GH standard curve (Figure 9.6). Displacement by a homogenate of the goldfish pituitary gland was also parallel to that of the carp GH standard curve (Figure 9.7). Serial dilutions of samples of perifusate collected following perifusion of goldfish pituitary fragments [Chapter 2] also result in displacement curves parallel to the carp GH standard curve (Figure 9.8). These results suggest that the newly validated carp GH RIA is specific for carp GH-like molecules, and indicate that the carp GH RIA is suitable for measuring serum levels of GH in the goldfish, the GH content in homogenates of the goldfish pituitary gland, and the GH levels in samples of medium collected during the _in_ _vitro_ incubation of the goldfish pituitary.

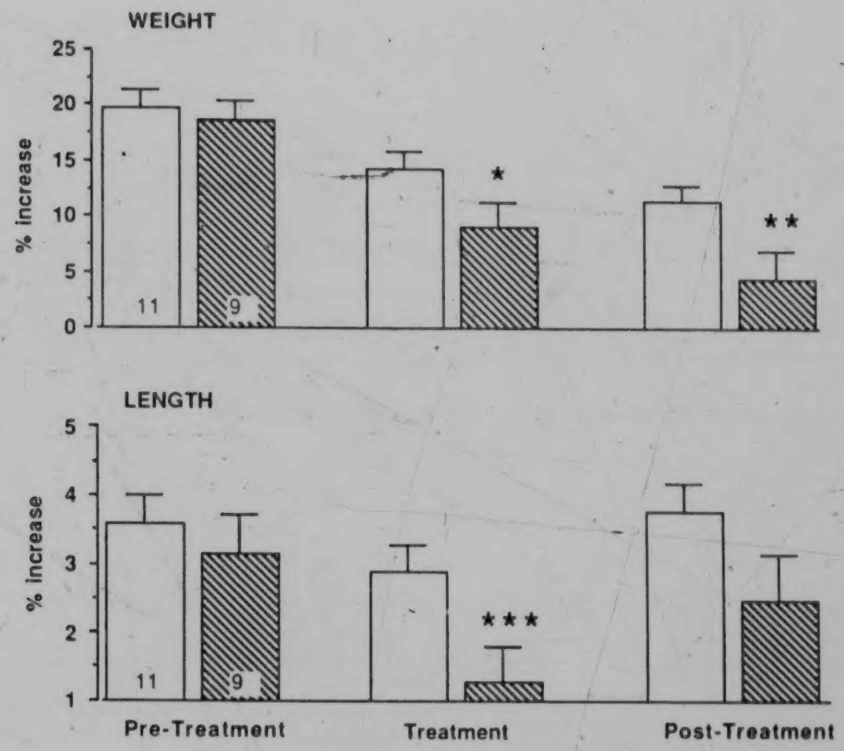

Figure 9.1. Growth rates of female goldfish injected during the treatment period with normal rabbit serum (open bars) or rabbit anti-carp GH serum (hatched bars). The growth rates are expressed as the % increase in body weight (top panel) or length (bottom panel) over each of the 12 day pre-treatment, treatment or post-treatment periods. Sample sizes are indicated along the abscissa. Significant differences in growth rate between the two groups are indicated (* p<0.1; ** p<0.05; *** p<0.01).

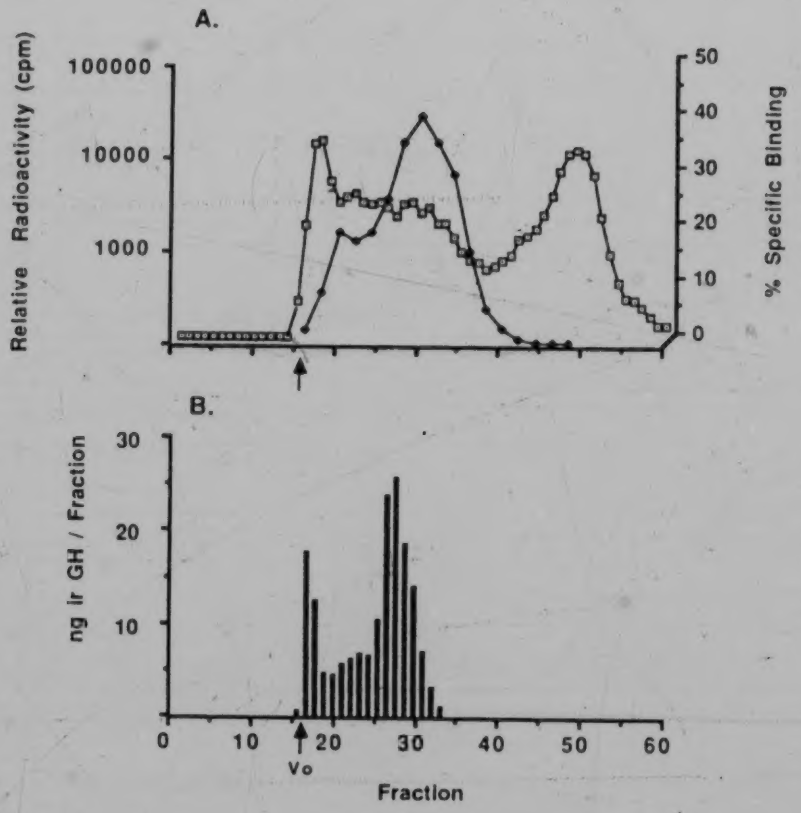

Figure 9.2. A. Profile of radioactivity (squares) following chromatography of the carp GH iodination reaction mixture on Sephadex G-100. The % of radioactivity in various fractions specifically bound by the antiserum is also indicated (diamonds). B. Profile of immunoreactive (ir) carp GH in 1 ml fractions collected following fractionation of "cold" carp GH on a Sephadex G-100 column. The void volume (Vo) in each column is indicated by the arrow.

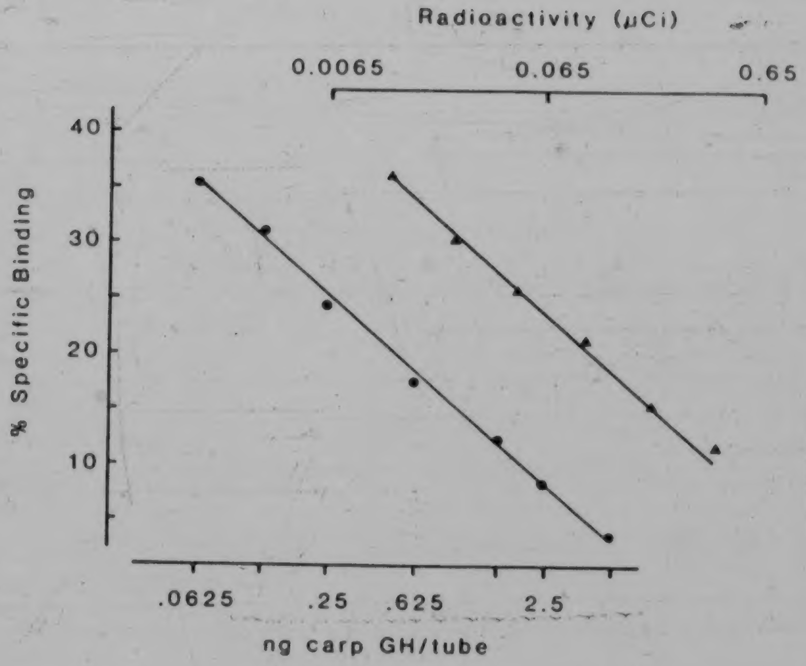

Figure 9.3. Displacement curves produced by increasing amounts of ^{125}I-carp GH (closed triangles) and by increasing amounts of the carp GH standard (closed circles). The specific activity of the ^{125}I-carp GH preparation was calculated from these curves as 248 μCi per μg carp GH.

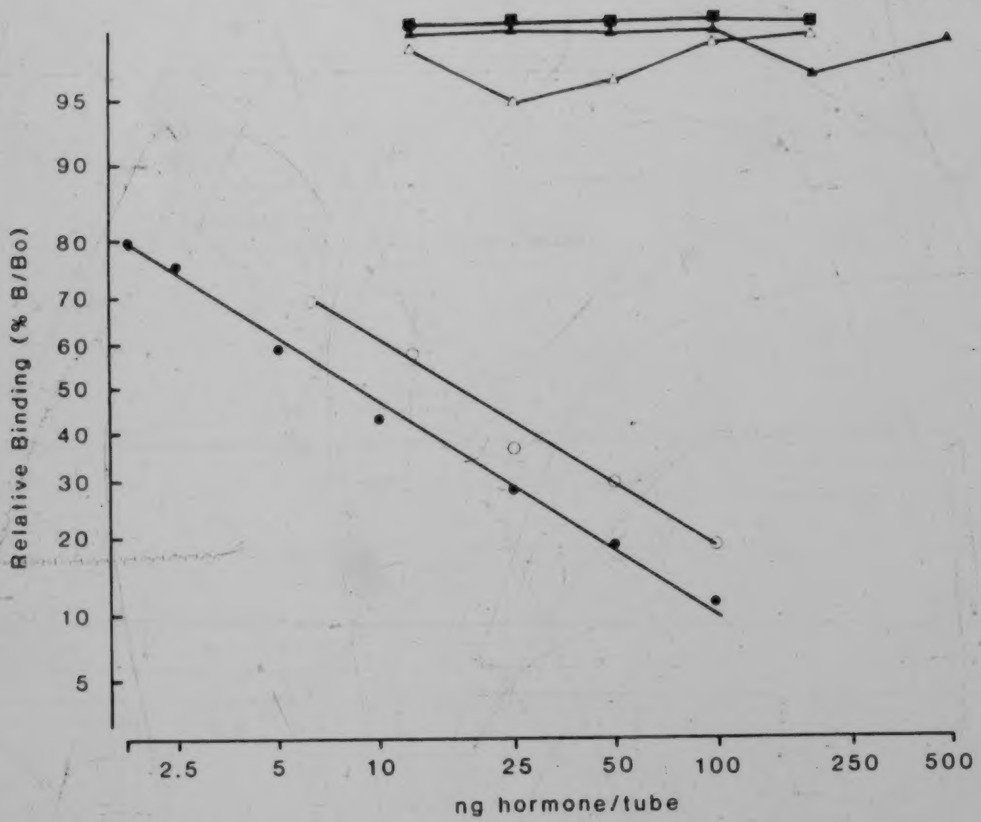

Figure 9.4. Displacement curves of various vertebrate pituitary hormone preparations in the carp GH RIA. Salmon prolactin (closed squares), salmon GH (closed squares), ovine prolactin (closed squares), bovine GH (closed squares), carp prolactin (closed triangles), and carp gonadotropin (open triangles) displayed little or no cross reactivity with the antiserum in the carp GH RIA. In contrast, the displacement curve produced by a carp GH preparation (open circles) described previously [Cook et al., 1983] was parallel to that of the carp GH standard (open circles) used in the RIA.

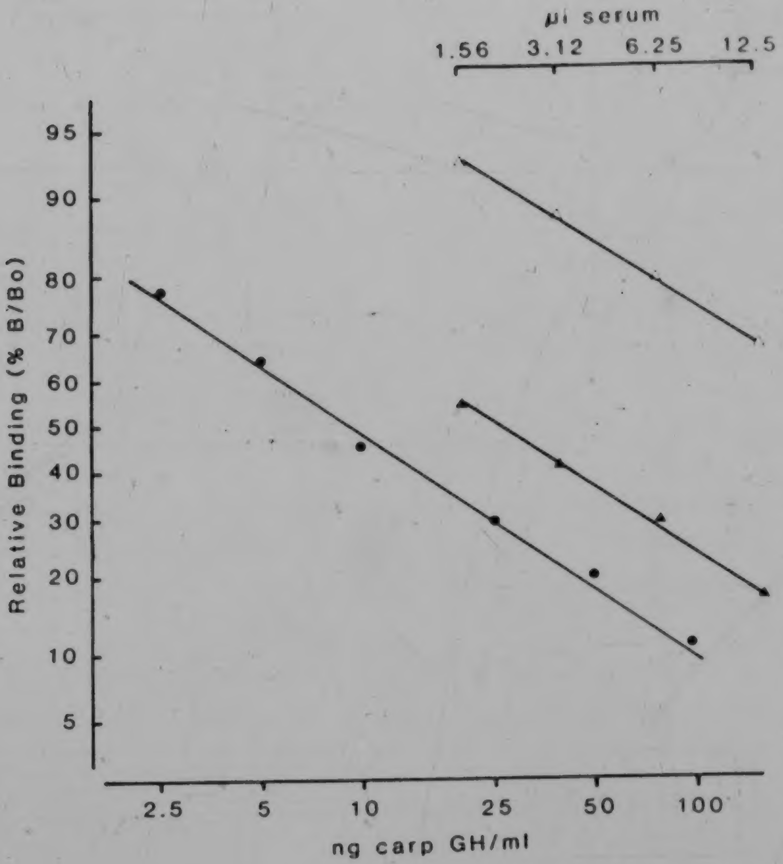

Figure 9.5. Comparison of RIA displacement curves produced by the carp GH standard (closed circles) and serial dilutions of serum from goldfish injected with saline (open triangles) or 0.1 μg/g mGnRH-A (closed triangles).

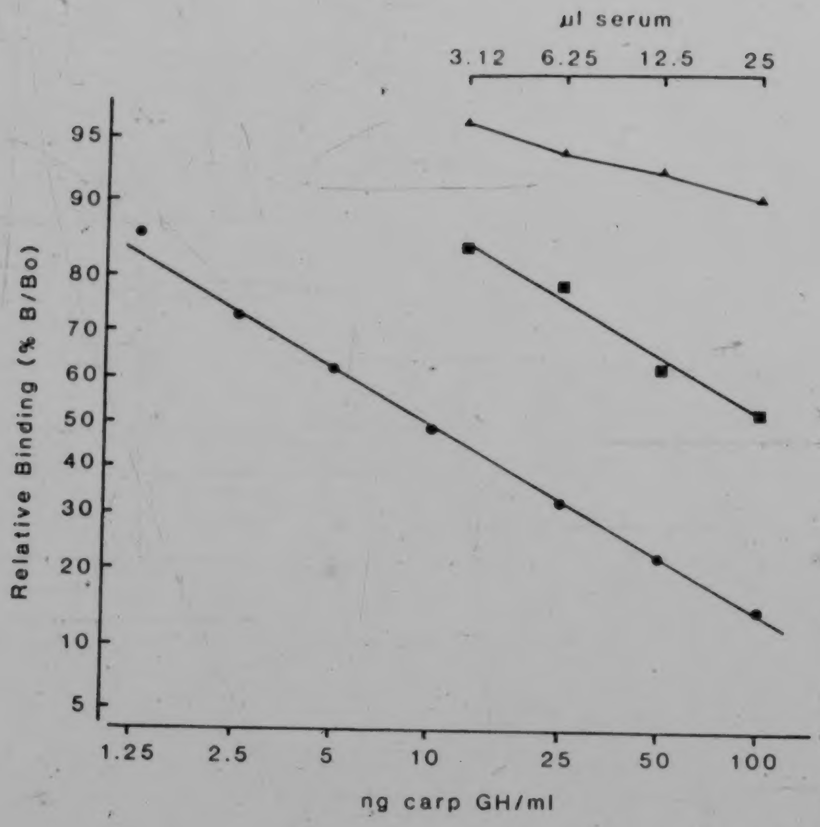

Figure 9.6. RIA displacement curves produced by the carp GH standard (closed circles) and serial dilutions of serum from hypophysectomized (closed triangles) or sham-operated (closed squares) goldfish.

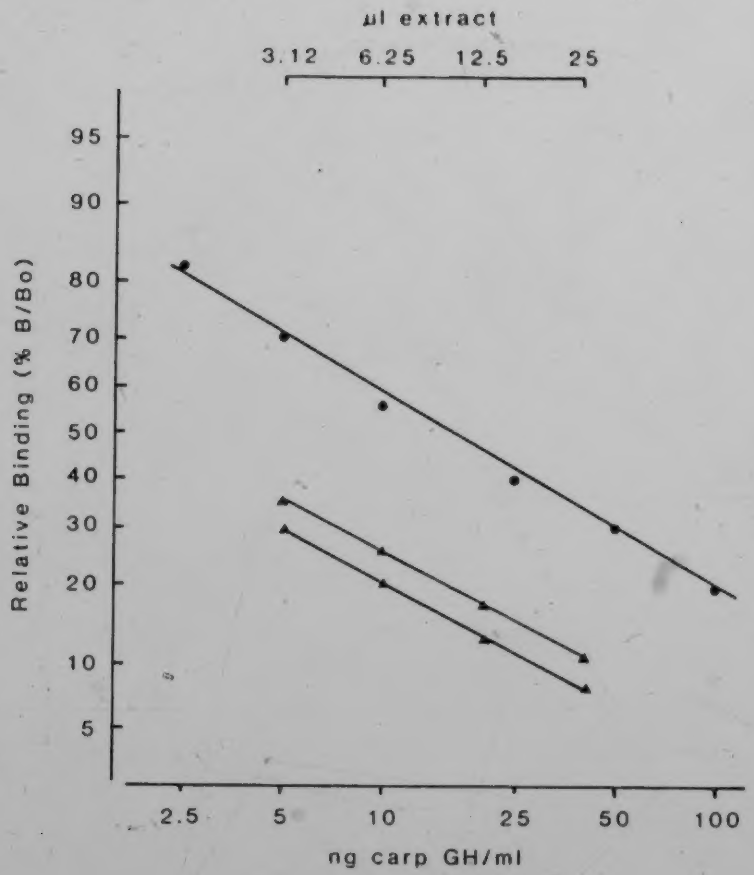

Figure 9.7. Displacement curves produced by the carp GH standard (closed circles) and serial dilutions of two preparations of homogenates of the goldfish pituitary gland (closed triangles) in the carp GH RIA. The pituitary homogenates were prepared by homogenizing each pituitary gland in 5 ml of assay buffer. This RIA was performed using the antiserum at a final dilution of 1:81,000.

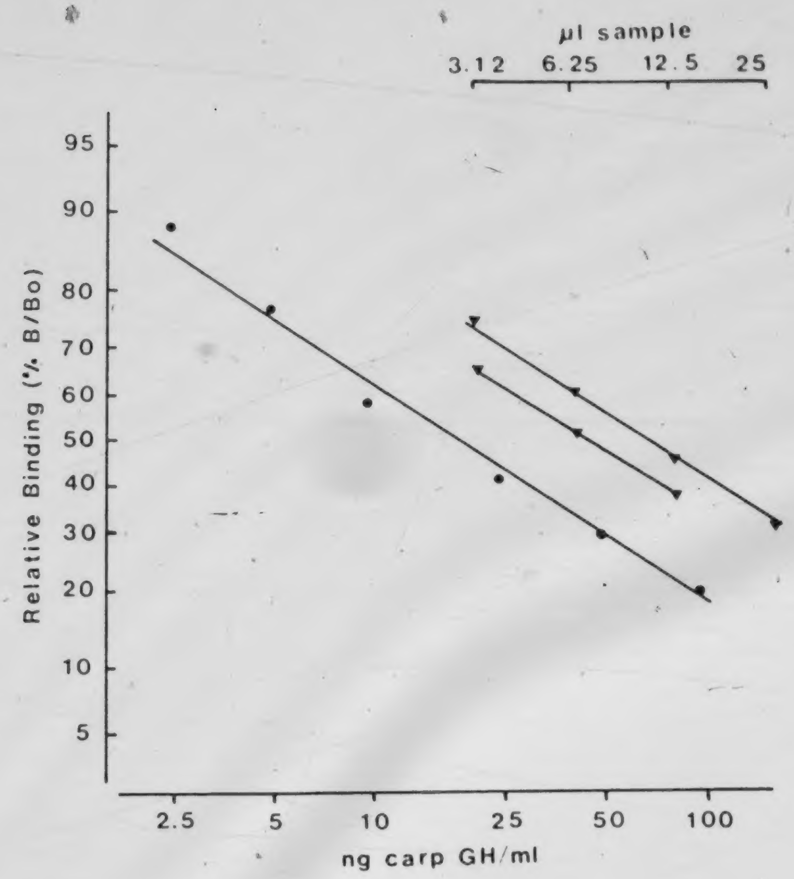

Figure 9.8. Displacement curves produced by the carp GH standard (closed circles) and serial dilutions of samples of perifusate collected during perifusion of goldfish pituitary fragments [see Chapter 2 for perifusion techniques] (closed triangles) in the carp GH RIA. This RIA was performed using the antiserum at a final dilution of 1:81,000.

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10. APPENDIX II

SOMATOSTATIN RADIOIMMUNOASSAY

The immunoreactive somatostatin (irSRIF) content of extracts of various goldfish tissues was measured by radioimunoassay (RIA) using a rabbit antiserum kindly provided by Dr. S.M. Sagar (Dept. of Neurology, Harvard Medical School, Boston, MA 02114). The antiserum was generated against synthetic SRIF-14 and is similar in potency and hormonal specificity to an antiserum used previously [Arnold *et al.*, 1982] to measure tissue levels of irSRIF in mammalian species [S. Sagar, personal communication]. Synthetic SRIF-14 (Peninsula Laboratories, Belmont CA) was used as the standard in this RIA, and the analog [Tyr^{1}]-SRIF-14 (Peninsula Laboratories) was used for iodination as described below.

Preparation of [^{125}I-Tyr^{1}]-SRIF-14

[Tyr^{1}]-SRIF-14 was iodinated using a modification of the method described by Patel and Reichlin [1978]. One mCi of Na-^{125}I in 50 μl of 0.5 M phosphate buffer (pH=7.4) was added to a conical vial containing 5 μg of [Tyr^{1}]-SRIF-14 in 10 μl of 0.01 N HCl. The reaction was initiated by the addition of 5 μg of Chloramine T in 5 μl of distilled water. Following 30 sec of gentle agitation, the reaction was terminated by dilution with the addition of 500 μl of 0.05 M phosphate buffer containing 10 % bovine serum albumin (BSA). The reaction mixture was fractionated by gel filtration on a 1 X 26 cm column of Sephadex G-25 (Pharmacia Chemicals, Dorval, Que). Prior to chromatography, the column was washed with 2 ml of 0.1 N acetic acid containing 5 % BSA to prevent adsorption of the labelled peptide to the column. The elution buffer was

0.1 N acetic acid containing 0.1 % BSA. One ml fractions of the eluate were collected using an automatic fraction collector, and the radioactivity in each fraction was monitored.

Three major peaks of radioactivity were separated during chromatography of the iodination mixture (Figure 10.1). The first peak elutes in the void volume (fractions 12-15), and is composed primarily of iodinated BSA [Patel and Reichlin, 1978]. The second peak (fractions 22-25) represents unreacted iodide, whereas [^{125}I-Tyr^{1}]-SRIF-14 elutes as a broad peak from fractions 30 through 55 [Patel and Reichlin, 1978]. The presence of [^{125}I-Tyr^{1}]-SRIF-14 in the third peak was confirmed by determining the relative amount of radioactivity in fractions from each peak bound by an excess of the antiserum (final dilution of antiserum = 1:250,000). Fractions from the first and second peaks displayed little or no binding to the antiserum (Figure 10.1). However, fractions in the third peak displayed significant binding to the antiserum, with the highest binding occurring in the fractions (46-52) corresponding to the shoulder of the third peak (Figure 10.1). Consequently, fractions from this region of the third peak were selected and pooled for use in the RIA. Previous studies have shown that preparation of [^{125}I-Tyr^{1}]-SRIF-14 in this manner results in the production of iodinated peptide with a specific activity of approximately 1000 µCi/µg peptide [Patel and Reichlin, 1978]. The pooled fraction of [^{125}I-Tyr^{1}]-SRIF-14 was stored undiluted at 4 °C, and was usually stable for up to 4 weeks under these storage conditions.

RIA Procedure

The assay buffer (PBS) consisted of 0.1 M NaH_2PO_4 (pH=7.4) containing 0.01 M EDTA, 0.05 M NaCl, 0.02 % sodium azide, and 0.1 % BSA. The RIA was performed using the antiserum at a final dilution of 1:500,000 and with approximately 10^4 cpm of [^{125}I-Tyr^1]-SRIF-14. Incubation tubes containing 200 µl of antiserum (1:200,000 intial dilution in PBS containing normal rabbit serum at a dilution of 1:40), 200 µl of [^{125}I-Tyr^1]-SRIF-14 appropriately diluted in PBS, and 100 µl of the standards (0 to 128 pg SRIF-14/tube) or 100 µl of appropriately diluted tissue extracts were incubated at 4 °C for 24 hr. Following this initial incubation, 200 µl of goat anti-rabbit serum (Calbiochem, La Jolla, CA) diluted 1:20 in PBS was added to each assay tube. After an additional incubation at 4 °C, the incubation tubes were centrifuged, the supernatant decanted, and radioactivity in the pellets counted. Non-specific binding (NSB) of [^{125}I-Tyr^1]-SRIF-14 was determined as the amount of radioactivity present in pellets of tubes incubated in the absence of antiserum; NSB determined in this way was similar to NSB measured in pellets of tubes containing antiserum incubated with an excess of the standard (<10 % of radioactivity originally present). Specific binding of [^{125}I-Tyr^1]-SRIF-14 to the antiserum in the absence of the SRIF-14 standard (corrected for NSB) was calculated as % of the total radioactivity originally present. The final dilution of antiserum used in the present RIA (1:500,000) resulted in specific binding of approximately 25 % of the [^{125}I-Tyr^1]-SRIF-14

The minimum sensitivity of the RIA, defined as the minimum concentration of SRIF-14 resulting in significant (p=0.01) displacement of the specifically bound [^{125}I-Tyr^{1}]-SRIF-14 [Reuter *et al.*, 1978], was calculated as 2 pg SRIF-14/tube. The concentration of SRIF-14 resulting in 50 % displacement of the specifically bound [^{125}I-Tyr^{1}]-SRIF-14 was calculated as 14.5 ± 0.68 pg/tube (mean ± SEM, n=8). Variability within the assay or between assays was assessed by determining the irSRIF content of tissue samples three times in a single assay or in three separate assays, respectively. The within assay variability for tissue samples containing 165 or 467 pg irSRIF/ml was calculated as % coefficient of variation (% CV) of 7.6 ± 1.4 and 6.8 ± 1.6 (X±SD), respectively; the between assay variability for each sample was calculated as % CV of 14.6 and 8.2, respectively. These intra- and inter-assay variabilities were within acceptable limits and similar to those reported previously for a SRIF RIA [Arnold *et al.*, 1982].

Assay Specificity

The hormonal specificity of the SRIF antiserum was tested by evaluating the crossreactivity of various vertebrate peptides with the antiserum (Figure 10.2). [Tyr^{1}]-SRIF-14 and mammalian SRIF-28 displayed significant crossreactivity in this RIA. Parallel line statistical analysis [Pekary, 1979] of the displacement curves for these peptides indicated the slopes of inhibition were parallel to that of SRIF-14. In contrast, the other peptides tested (Figure 10.2) displayed no crossreactivity with the antiserum at concentrations up to 12.8 ng/tube. These results suggest that the antiserum is specific for peptides very similar in structure to SRIF-14. However, the observation that mammalian

SRIF-28 also crossreacts with the antiserum indicates that longer SRIF peptides containing the SRIF-14 sequence would also be detected in the present RIA.

Previous studies in a variety of teleost species have demonstrated irSRIF in several tissues, including the brain, pituitary, pancreas and intestine [Holmgren _et_ _al_., 1982; Kah _et_ _al_., 1982; Langer _et_ _al_., 1979; Olivereau _et_ _al_., 1984; Vigh-Teichman _et_ _al_., 1983]. The suitability of the present RIA for measuring the irSRIF content in extracts of various goldfish tissues was assessed by comparing the slopes of inhibition of serial dilutions of tissue extracts with that of the SRIF-14 standard using the statistical program described by Pekary [1979]. Extracts of the pituitary and various brain regions of the goldfish were prepared using the procedures described previously [Chapter 3]. SRIF-14 like immunoreactivity was detected in extracts of the pituitary gland (Figure 10.3) and brain areas (Figure 10.4); the slopes of inhibition of serial dilutions of these extracts were parallel to that of the SRIF-14 standard. Extracts of the anterior portion of the small intestine, hepato-pancreatic tissue (liver containing pancreatic tissue), ovary, and muscle of the goldfish were also prepared by processing 5 to 10 mg wet weight of these tissues using the extraction procedure described for brain tissue [Chapter 3]. irSRIF was detected in extracts of the goldfish intestine and hepato-pancreatic tissue (Figure 10.5); the slopes of inhibition of these extracts were parallel to the SRIF-14 standard curve. In contrast, irSRIF was not detected in extracts of the goldfish ovary or muscle. The demonstration of parallelism between serial dilutions of the tissue extracts and the SRIF-14 standard curve indicates that the present RIA is suitable for the measurement of irSRIF content in extracts of the goldfish pituitary, brain, intestine and pancreas.

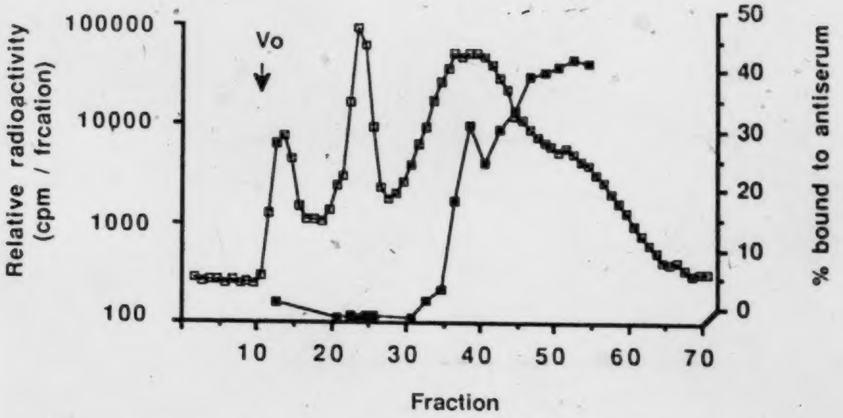

Figure 10.1. Profile of radioactivity (open squares) following fractionation of the [Tyr¹]-SRIF-14 iodination reaction mixture on a Sephadex G-25 chromatography column. The % of radioactivity in various fractions specifically bound by the antiserum is also indicated (closed squares).

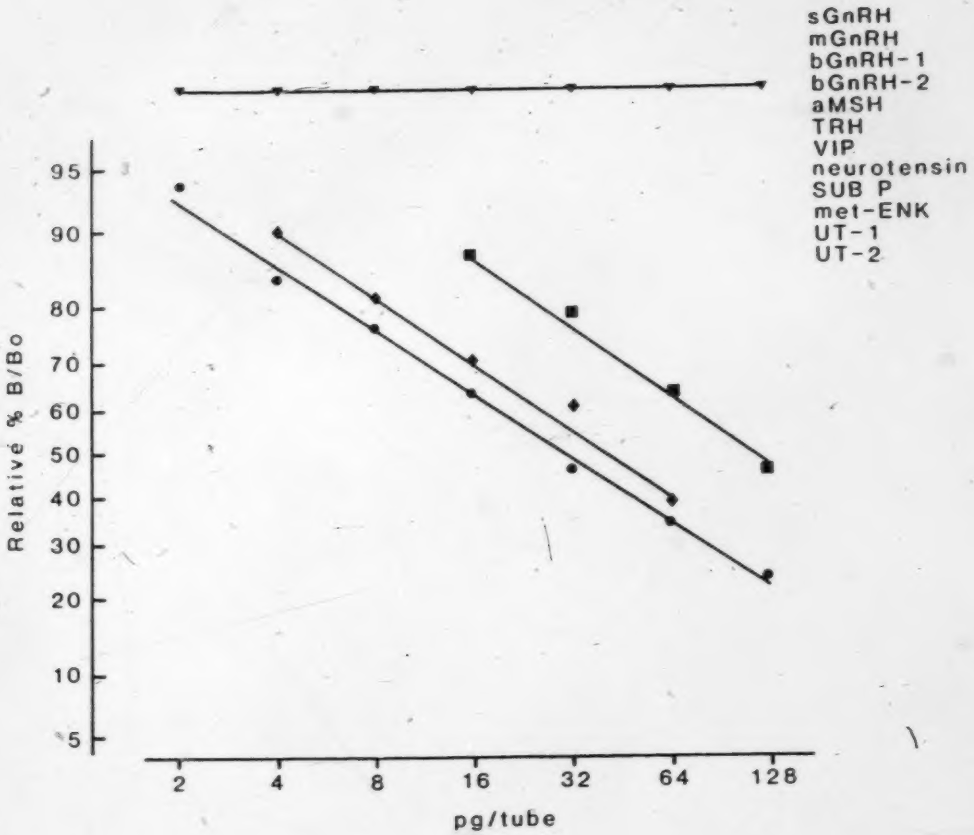

Figure 10.2. Displacement curves of various synthetic vertebrate peptides in the SRIF RIA. Abbreviations: SRIF - somatostatin; GnRH - gonadotropin releasing hormone; sGnRH - salmon GnRH; mGnRH - mammalian GnRH; bGnRH - chicken GnRH; aMSH - α melanocyte stimulating hormone; TRH - thyrotropin releasing hormone; VIP - vasoactive intestinal polypeptide; SUB P - substance P; met-ENK - met-enkephalin; UT - urotensin.

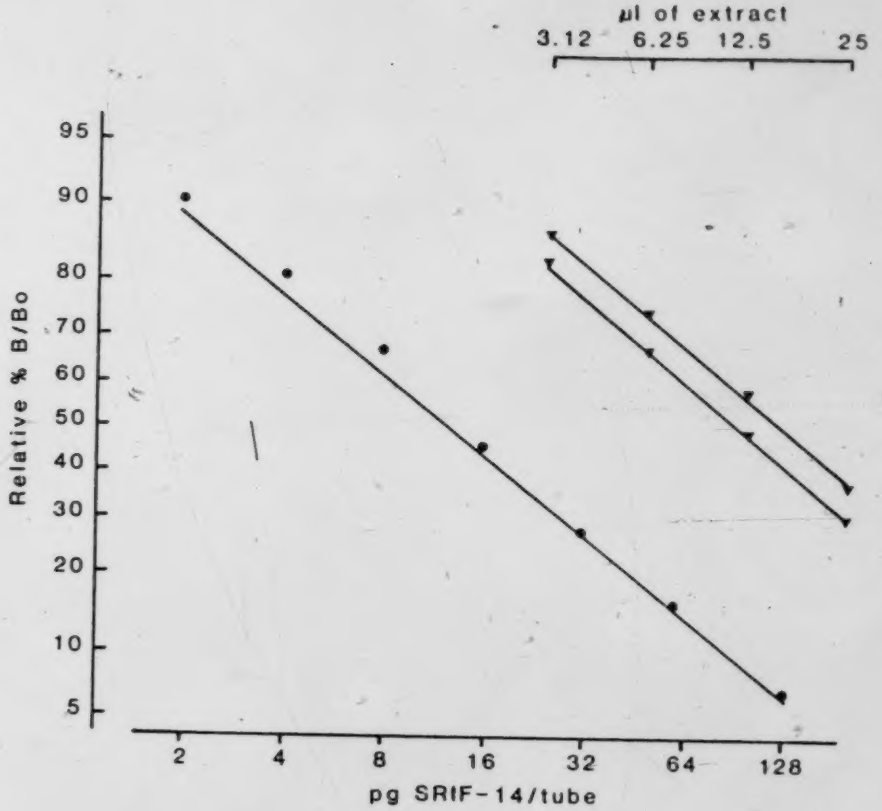

Figure 10.3. Comparison of RIA displacement curves produced by the SRIF-14 standard (circles) and serial dilutions of extracts of the pituitary glands from two goldfish.

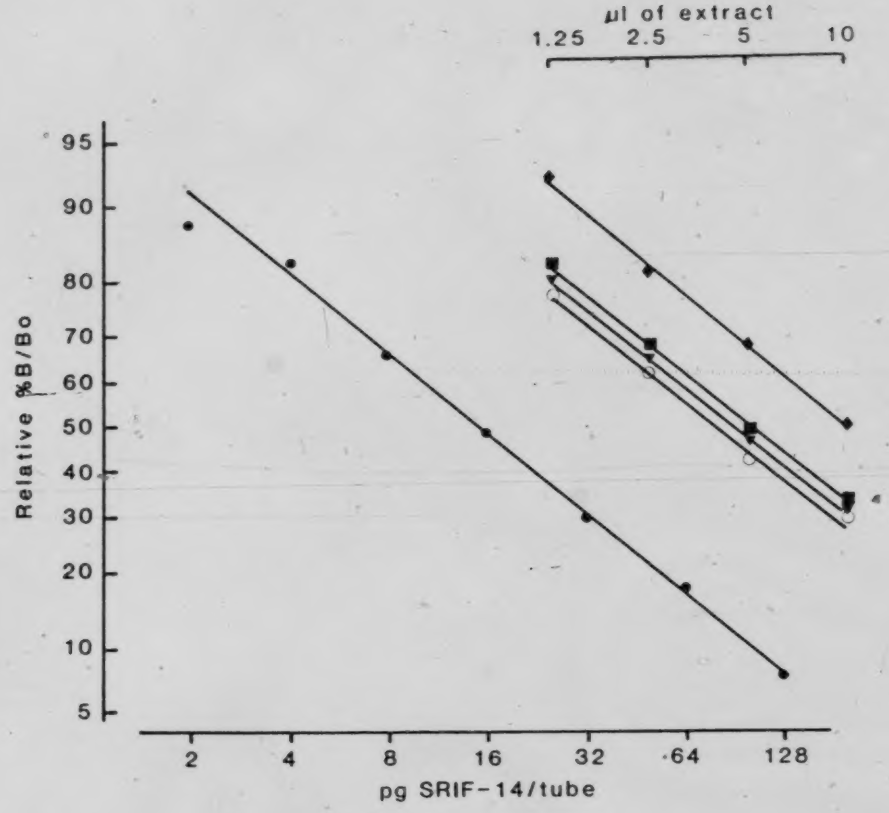

Figure 10.4. Comparison of RIA displacement curves produced by the SRIF-14 standard (closed circles) and serial dilutions of extracts of the goldfish telencephalon (open circles), medulla+brain stem (triangles), optic tectum+thalamus (squares), and hypothalamus (diamonds).

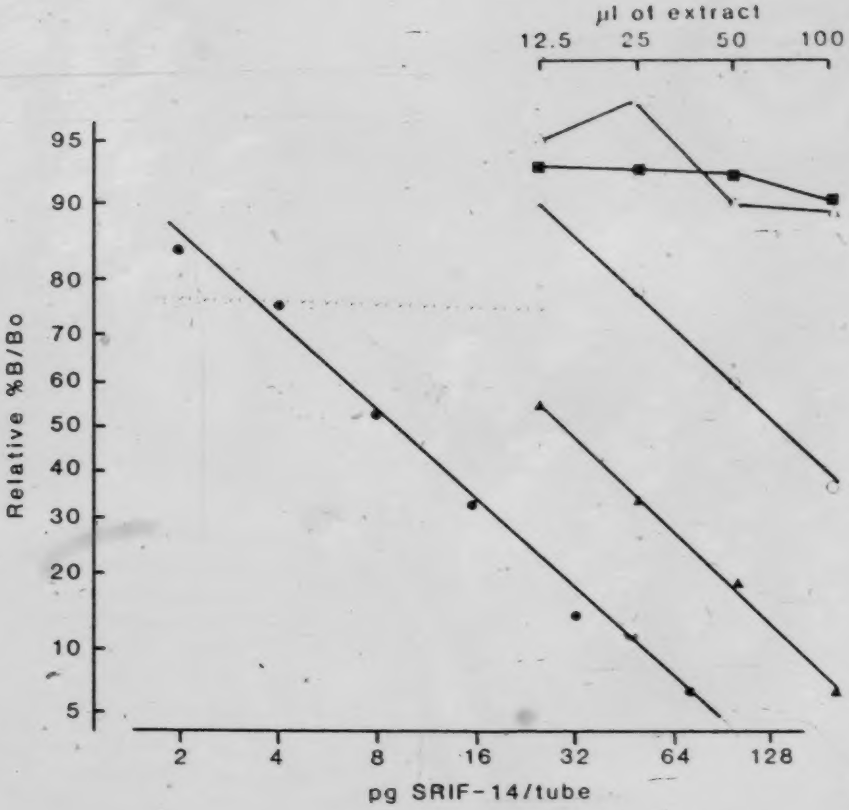

Figure 10.5. Comparison of RIA displacement curves produced by the SRIF-14 standard (closed circles) and serial dilutions of extracts of the goldfish anterior intestine (closed triangles), hepato-pancreatic tissue (open circles), muscle (squares), and ovary (open triangles).

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